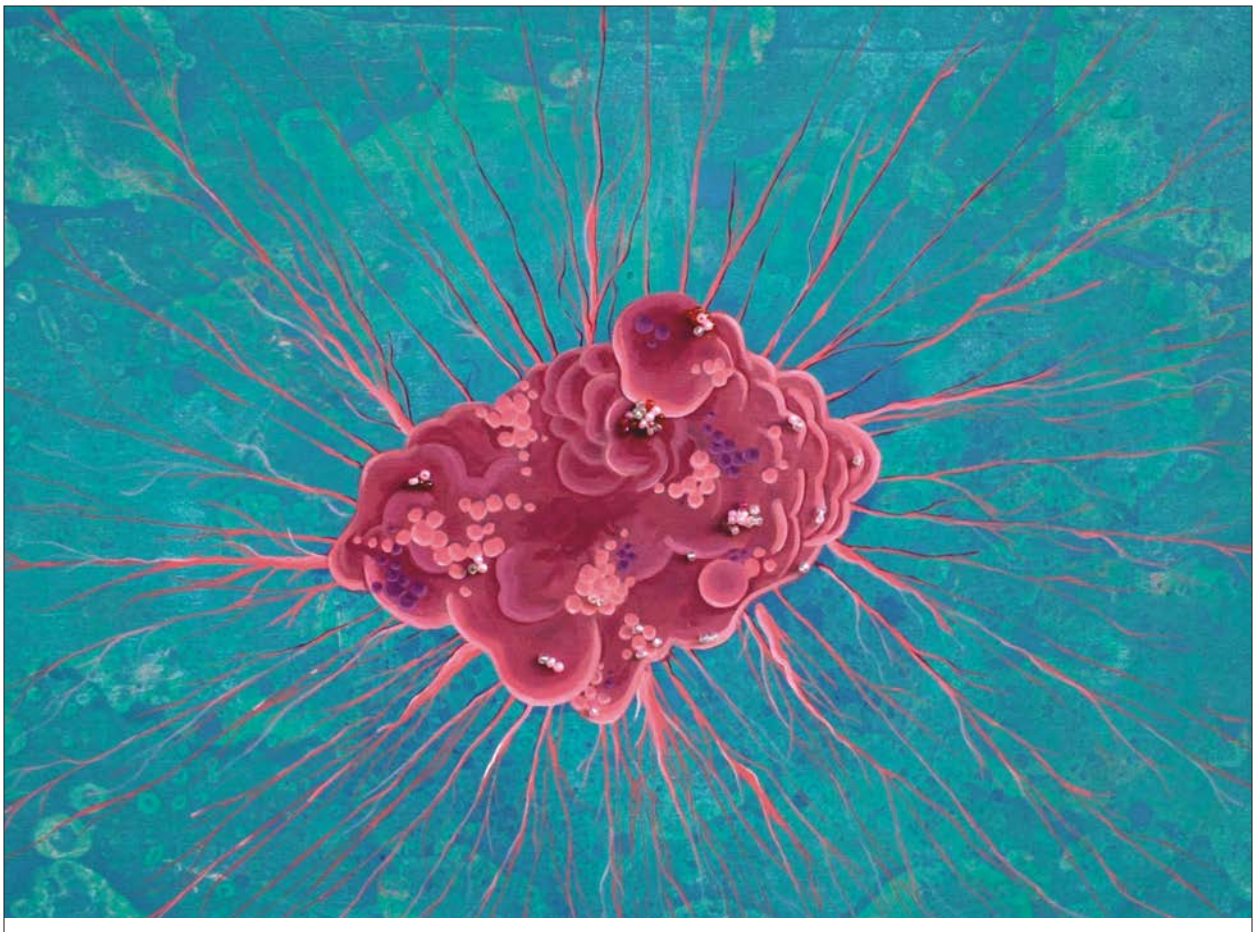

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Chemopreventive Effect of *Acanthus ilicifolius* Extract on Modulating Antioxidants, Lipid Peroxidation and Membrane Bound Enzymes in Diethyl Nitrosamine Induced Liver Carcinogenesis

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ABSTRACT

Acanthus ilicifolius (Acanthaceae) has received considerable attention due to its wide range of traditional usage in Indian system of medicine. The chemopreventive effect of ethanol extracts of *Acanthus ilicifolius* (AIEE) on N-nitrosodiethylamine (DEN, 200 mg kg^{-1})-induced experimental liver tumour was investigated in male Wistar rats. Experimental rats were orally treated with AIEE (400 mg kg^{-1} b.wt.) for 1 week before the injection of diethyl nitrosamine and continued to be 14 weeks. The changes of lipid peroxidation, antioxidants and membrane bound enzymes were studied in AIEE treated experimental and control rats. The result indicates AIEE treatment effectively suppressed liver tumour induced with DEN as revealed by decrease in the levels of extend of alanine transaminase, aspartate transaminase, alkaline phosphatase, lipid peroxidase, glutathione peroxidase and glutathione-S-transferase with a concomitant increase in enzymatic antioxidant (superoxide dismutase and catalase) levels when compared to those in liver tumour bearing rats. The result of the study concludes significant chemopreventive effect of AIEE against DEN induced liver tumour.

Key words: Adenosine triphosphatase, *Acanthus ilicifolius*, diethyl nitrosamine, glycoprotein, hepatocellular carcinoma, mangrove

INTRODUCTION

Liver cancer is the fifth most common cancer in worldwide representing 83%. About 12.5% of all deaths are caused by cancer that is more than the percentage of death caused by HIV/AIDS, tuberculosis and malaria (Jemal *et al*., 2007). The synchronous occurrence of HCC may be due to different risk factors, such as chronic viral hepatitis B or hepatitis C infection, aflatoxin exposure, alcohol consumption and iron overload (Zhou *et al*., 2000). The other relative factors which lead to HCC are metabolic disorders, including glycogen storage disease (Rajamanickam *et al*., 2014). The intracellular accumulation of iron due to haemochromatosis may cause oxidative stress and DNA damage (Tsukuma *et al*., 1993) thereby contributing the factor for hepato carcinogenesis. Moreover, there is a growing understanding of the molecular mechanisms inducing hepato carcinogenesis, which almost never occurs in healthy liver, the cancer risk increases sharply in response to chronic liver injury in the cirrhosis stage (Ueno *et al*., 2001). Hepatitis is associated with liver cell necrosis, inflammation, regeneration and fibrosis, which may lead to cirrhosis. Chronic hepatitis is characterized by repetitive cycles of necrosis and regeneration, which facilitates successive

acquisition of genomic alterations. These may escape repair mechanisms and ultimately lead to the development of HCC through monoclonal expansion (Cragg *et al*., 1997). Role of natural products in the cure of human diseases have been recognized since ancient times. An analysis of a number of chemotherapeutic agents and their sources indicates that over 60% of approval drugs derived from the natural compounds. Several plant derived compounds are currently successfully employed in cancer treatment. One of the most significant examples is the vinca alkaloids isolated from *Catharanthus roseus* that inhibitors microtubules assembly that induces self-association in to coiled spiral aggregates (Noble, 1990).

Development of novel agents from natural sources presents some obstacles. *Acanthus ilicifolius* (Family: Acanthaceae) is commonly known as holy mangrove and used as a traditional medicine to treat pain and inflammation, rheumatism, paralysis, neuralgia, asthma, snake bite, leucorrhoea and debility effects on the Southeast coast of India. Studies reported that, *A. ilicifolius* extracts has antimicrobial, anti-oxidant, analgesic, anti-inflammatory, anti-plasmodial, anti-viral, hemolytic, hepato-protective, anti-ulcer, anti-leishmanial, anti-cancer activities and prevent DNA alteration in carcinogen animals models (Agshikar *et al*., 1979; Kapil *et al*., 1994; Babu *et al*., 2001, 2002; Chakraborty *et al*., 2007; Khajure and Rathod, 2010; Ravikumar *et al*., 2011; Thirunavukkarasu *et al*., 2011; Nizamuddin *et al*., 2011; Singh and Aeri, 2013). Metabolites such as gallic acid, quercetin, lupeol, benzoquinone, lyoniresinol glucopyranoside, tetrahydrofuran-9-O-glucopyranoside, campesterol, cinnamate, saturated fatty acid and essential oils, alkanes and stigmasterol reported in *A. ilicifolius* (Kanchanapoom *et al*., 2001; Goswami *et al*., 2004; Nebula *et al*., 2013; Selvaraj *et al*., 2014; Satyavani *et al*., 2015). Earlier potential cytotoxic effect of ethanolic extract of *A. ilicifolius* on HepG2 cells have been reported (Rajamanickam *et al*., 2014). On the basis of previous scientific reports, the present study investigated the chemo-preventive effect of *A. ilicifolius* on diethyl nitrosamine induced hepatocellular carcinoma in animal models.

MATERIALS AND METHODS

Chemicals: Nitro Blue Tetrazolium (NBT), Ethylene Diamine Tetraacetic Acid (EDTA), Trichloro Acetic Acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dintiro benzene (CDNB), 5,5'-dithio-bis (2-nitrobenzoic acid), glutathione (reduced), glutathione (oxidized), Diethyl nitrosamine (DEN) and L-ascorbic acid were purchased from Sigma Aldrich (USA). All other chemicals used were of analytical grade and were obtained from Hi Media (Mumbai, India).

Plant material: Fresh leaves of *A. ilicifolius* were collected from Parangipettai coastal Village, Tamil Nadu, India during the months of January, 2008. The plant species was identified, authenticated and deposited in the herbarium of C.A.S. in Marine Biology, Annamalai University, Parangipettai, India (Voucher No.: AUCASMB 01/2008).

Extraction: Leaves of *A. ilicifolius* were shade dried and powdered with a mechanical grinder and stored in an airtight container. The powder material of leaves (1 kg) was extracted with ethanol (2500 mL) by Soxhlet method for 3 h. The solvent was completely removed by drying and ethanolic extract of *A. ilicifolius* (AIEE) was prepared freshly in distilled water and used further experimental studies.

Experimental animals: Male albino rats of Wistar strain approximately (200-250 g) were used in this study. They were healthy rats procured from Central Animal House Facility, Rajah Muthiah Medical College and Hospital, Annamalai University, Tamil Nadu, India. The rats were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (12 h light/dark cycles) throughout the experimental period. All the rats were fed with standard pellet diet (Hindustan Co. Pvt., Mumbai, India) and water *ad libitum*. They were acclimatization to the environment for 1 week prior to experimental use. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Rats (CPCSEA) and Institutional Animal Ethical Committee (Reg. 169/CPCSEA/1999).

Toxicity and dose fixation studies of AIEE: The rats were divided into different groups and each group administrated orally with AIEE at the dose level of 50, 300, 1000, 2000 and 4000 mg kg^{-1}/b.wt. The rats were observed up to 48 h for behavioral change and mortality. The absence of any adverse side effect and mortality on administration of an acute dose 4000 mg kg^{-1} of AIEE clearly indicated the non-toxic nature of the plant extract. Based on the non-toxic nature of the extract 400 mg kg^{-1} b.wt., dose was selected for further studies.

Cancer induction: Animal received a single intraperitoneal injection of N-Nitrosodiethylamine (DEN) at a dose of (200 mg kg^{-1} b.wt.) in saline to induce liver cancer. Two weeks after administration of DEN, phenobarbital at a concentration of 0.05% was incorporated into rat chow for upto 14 successive weeks to promote the cancer.

Experimental design: There were 28 rats totally used in the experiment (14 normal rats+14 cancer bearing rats). The AIEE orally administrated to the experimental rats using intragastric tube. Body weights of the rats were recorded and they were divided into 4 groups of 6 rats each as follows:

Group 1 : Control rats received normal saline (0.9%)
Group 2 : Cancer control rats
Group 3 : Cancer bearing rats treated with AIEE (400 mg kg^{-1} b.wt.) for 28 days
Group 4 : Normal rats received orally AIEE (400 mg kg^{-1} b.wt.) for 28 days

At the end of the experimental period, all the animals were anesthetized with thiopentone sodium (50 mg kg^{-1}), which were sacrificed by cervical decapitation. Animals were starved overnight before sacrifice. Blood was collected and the serum was separated by centrifugation. The organs such as the liver and kidney tissues were dissected out and washed 2-3 times with saline and known weight of liver tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was subjected to differential centrifugation. The cell organelles such as mitochondria, microsomes and cytosolic fractions were isolated. Total homogenate and sub cellular fractions were used for the assay of the following parameters in serum, plasma, liver and kidney samples.

Determination of tumour weight of liver and kidney: Tumour weight was estimated according to the method of Geran *et al*. (1972). The resultant solid tumour was considered to be

prelate ellipsoid with one long axis and two short axis. The two short axis were measured with a vernier caliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by two:

$$\text{Tumor weight (g)} = \frac{\text{Length (cm)} \times \text{Width (cm}^2\text{)}}{2}$$

Biochemical parameters using serum samples

Estimation of liver marker enzymes: The activities of aspartate transaminase (AST), alanine transaminase (ALT), 5'-Nucleotidase (Luly *et al*., 1972), g-glutamyl transpeptidase (Rosalki and Rau, 1972), lactate dehydrogenase (King, 1965) were assayed in the serum samples of experimental rats.

Estimation of glycoprotein: To the known volume of serum was mixed with 2.0 mL of alcohol, left for 20 min and centrifuged. To the precipitate, 3.0 mL of 3 N HCl was added and hydrolysis for 4 h at 100°C in water bath for the estimation of hexose (Niebes, 1972), hexosamine (Wagner *et al*., 1984) and sialic acid level (Warren, 1959).

Estimation of antioxidants: The activity of serum superoxide dismutase (Marklund and Marklund, 1974), catalase (Sinha, 1972), glutathione peroxidase (Rotruck *et al*., 1973), glutathione (Moron *et al*., 1979), ascorbic acid (Omaye *et al*., 1979), vitamin E (Desai, 1984) was determined.

Biochemical parameters using liver tissue samples

Determination of Lipid profile: Total lipid was extracted and quantified according to the method of Folch *et al*. (1957). The lipid extract obtained as described earlier was re-dissolved in chloroform and methanol in the ratio of 2:1 and mixed with 1.0 mL of potassium chloride (0.1 N). The contents were shaken well. The upper aqueous phase containing ganglioside and other water soluble compounds were discarded. The lower phase was again washed three times with 2.0 mL of Folch's reagent and the upper aqueous phase was aspirated. The lower chloroform phase was made upto a known volume and aliquots taken for the analysis of total cholesterol (Parekh and Jung, 1970), free cholesterol (Hron and Menahan, 1981), triglyceride (Schenk *et al*., 1975), phospholipids (Rouser *et al*., 1970) and free fatty acids (Rice, 1970).

Estimation of carbohydrate metabolizing enzymes: Hexokinase, glucose-6-phosphatase, aldolase, phosphoglucoisomerase and fructose-1,6 biphosphatase levels were estimated. For these assays, 1 g of fresh/frozen liver was chopped and homogenized in ice-cold sucrose (15 mL, 250 mM) for 2 min, centrifuged at 10000×g for 30 min and the supernatant was used as the enzymatic source (Brandstrup *et al*., 1957; Horrocks *et al*., 1963; King, 1965; Ohkawa *et al*., 1979).

Biochemical parameters using liver and kidney tissue samples: The nucleic acids were extracted by the method of Schneider (1957). A known amount of tissues was homogenized in 5.0 mL of ice-cold distilled water using Potter-Elvehjem homogenizer with a teflon pestle. The 5.0 mL of 5% TCA was added to the homogenate and this was kept in ice for 30 min to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. Then it was treated with 95%

ethanol to remove lipids. The final precipitate was heated at 90ºC for 15 min with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA (Burton, 1956) and RNA (Rawal *et al*., 1977).

Estimation of adenosine triphosphatase: Adenosine triphosphatase catalyze the conversion of adenosine triphosphate in to adenosine diphosphate. During the conversion, one molecule of phosphate was liberated. The inorganic phosphate was estimated according to the method of Fiske and Subbarow (1925). The proteins were precipitated with trichloroacetic acid. The free filtrate reacts with acid molybdate solution to form phospho molybdic acid which is reduced by the addition of 1-amino 2-naphthol-4-sulphonic acid (ANSA) to produce blue colour. The intensity of the colour is proportional to the amount of phosphate present. Further the filtrate was used to estimate Na^+, K^+-ATPase (Kuijpers and Bonting, 1970), Ca^{2+}-ATPase (Hjerten and Pan, 1983) and Mg^{2+} ATPase (Ohnishi, 1962; Ohnishi *et al*., 1982).

Statistical analysis: Values were expressed as Mean±SD for six rats in the each group and statistically significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Turkey's test using Graph Pad Instat software (USA). A value of $p<0.05$ was considered statistically significant.

RESULTS

Effect of AIEE on physiological changes: In the present study, AIEE treated control rats showed remarkable changes in their behavior when compared to normal rats. Cancer bearing rats were comparatively less than AIEE treated cancer bearing rats. The AIEE treatment to the group 4 rats was found to be quite active and healthy in behaviour. There were no significant difference in food and water consumption in AIEE treated rats when compared to the control. Greyish white hyper plastic nodules were obtained from the entire animal treated with DEN. It was easily identified from the reddish brown surrounding liver tissues. In carcinogen untreated group, the total number of nodules was 192 whereas, in AIEE treated group, it was decreased to 58.

Body weights were significantly decreased in untreated cancer bearing rats when compared to normal control ($p<0.001$), which was considerably increased in AIEE treated rats ($p<0.01$). On the other hand, liver weights were increased slightly in group 3 rats ($p<0.01$) when compared to the control. There was a mild decrease in the liver weight of group 4. However, no statistical differences were found in liver and kidney weight during treatment (Table 1).

Effect of AIEE on liver function enzymes: There was a major ($p<0.001$) increase in serum ALT, AST, ACP, ALP, 5'-ND and LDH in cancer bearing rats. In AIEE treated cancer bearing rats, the levels of ALT, ACP, ALP and 5'-ND were highly reduced ($p<0.001$). The levels of AST, ALT, ACP and ALP, 5' ND and LDH were retained nearer to normal in AIEE treated normal rats (Fig. 1).

Effect of AIEE on glycoprotein level: Figure 2 shows the levels of glycoproteins such as hexose, hexosamine and sialic acids in serum of control and experimental rats. The increased levels of hexose, hexosamine and sialic acids were observed in cancer bearing rats when compared to the control rats ($p<0.001$). In AIEE treated cancer bearing rats the levels of hexose, hexosamine and sialic acids were decreased. There were no remarkable changes observed in AIEE treated control rats when compared with control.

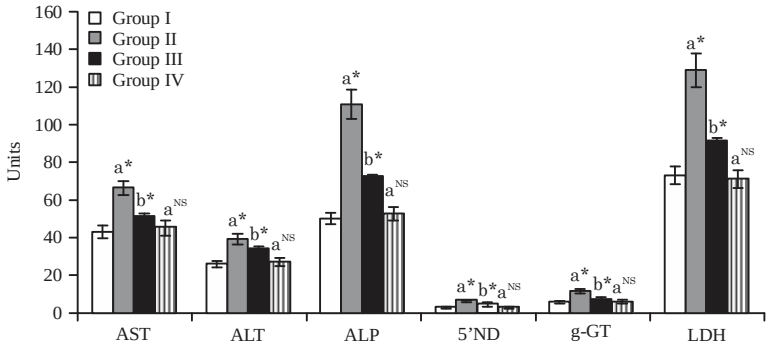

Fig. 1: Effect of AIEE on the activities of liver marker enzymes in serum of experimental rats. Each value represents Mean±SD for six animals. [a]Group 1: compared with Group 2 and 4, [b]Group 3 compared with Group 2, NS: Non significant, *p<0.001, AST-µ: Moles of pyruvate liberated/mg protein/min, ALT-µ: Moles of pyruvate liberated/mg protein/min, ALP-µ: Moles of p-nitrophenol liberated/mg protein/min

Table 1: Effect of AIEE on physical parameters, carbohydrate metabolic enzymes and lipid peroxidation enzymes in control and experimental rats

Parameters	Normal control	Cancer control	Cancer bearing rats+AIEE	Normal rats+AIEE
Physical parameters				
Body weight (g)	153.22±11.81	126.67±10.62^{a*}	163.46±11.01^{b*}	154.67±12.23^{ans}
Liver	6.59±0.44	7.84±0.56$^{a\#}$	7.01±0.49^{bns}	6.60±0.43^{ans}
Kidney	4.69±0.28	5.76±0.31^{a*}	5.03±0.38^{bns}	4.78±0.29^{ans}
Carbohydrate metabolite enzymes				
Hexokinase (n moles of glucose-6-phosphate liberated/mg protein/min)	12±0.12	26±0.25^{a*}	16±0.18^{b**}	13±0.13^{ans}
Phosphoglucoisomerase (n moles of fructose liberated/mg protein/min)	25±0.11	43±0.23^{a*}	31±0.12^{b*}	27±0.12^{ans}
Aldolase (n moles of glyceraldehydes, liberated/mg protein/min)	23±0.12	37±0.13^{a*}	28±0.16^{b**}	21±0.10^{ans}
Glu-6-Phosphatase (n moles of inorganic phosphate, liberated/mg protein/min)	24±0.13	14±0.12^{a*}	19±0.14^{b*}	18±0.12^{ans}
Fructose-1, 6 diphosphatase (n moles of inorganic, phosphate, liberated/mg protein/min)	33±0.21	19±0.16^{a*}	26±0.12^{b**}	32±0.02^{ans}
Lipid peroxidation (nmole of TABRS formed mg of protein/min)				
Basal	1.6±0.02	3.6±0.23^{a}	1.8±0.21^{b**}	1.5±0.04^{ans}
Hydrogen peroxide	1.4±0.03	3.1±0.14^{a}	1.6±0.31^{b**}	1.6±0.06^{ans}
Ascorbic acid	3.2±0.06	6.2±0.15^{a}	5.6±0.18^{b**}	3.6±0.02^{ans}
Ferrous sulphate	5.8±0.02	11.8±0.24^{a}	9.4±0.16^{b**}	6.1±0.03^{ans}

Each value represents Mean±SD of six animals, a: Group 2, 4 compared with group1, b: Group 3 compared with Group 2, #: p<0.01 *p<0.001, ns: Not significant

Effect of AIEE on antioxidant enzyme levels: Figure 3 represented the activities of superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin C and vitamin E in the serum of control and experimental rats. There was a decreased activity of superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin C and vitamin E observed in cancer bearing rats. The levels of these antioxidants were increased significantly in AIEE treated cancer bearing rats. No remarkable changes were observed in AIEE treated normal rats.

Effect of AIEE on lipid profile: Figure 4 showed that the lipid profile in the serum of control and experimental rats. Total cholesterol, free cholesterol, phospholipids, free fatty acids and triglycerides levels were found to be significantly increased in cancer bearing control rats, when compared to control rats. In AIEE treatment to the cancer bearing rats, the levels of lipid profile

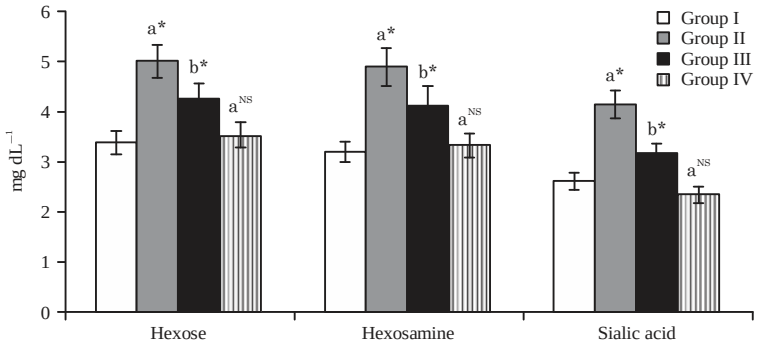

Fig. 2: Level of glycoprotein's in serum of experimental rats. Each value represents Mean±SD for six animals. [a]Group 1 compared with Group 2 and 4, [b]Group 3 compared with Group 2, NS: Non significant; *p<0.001. 5'ND-nmoles of phosphate, Liberated/mg protein/min, γ-GT-IU/L, LDH-μ moles of pyruvate liberated/mg protein/min

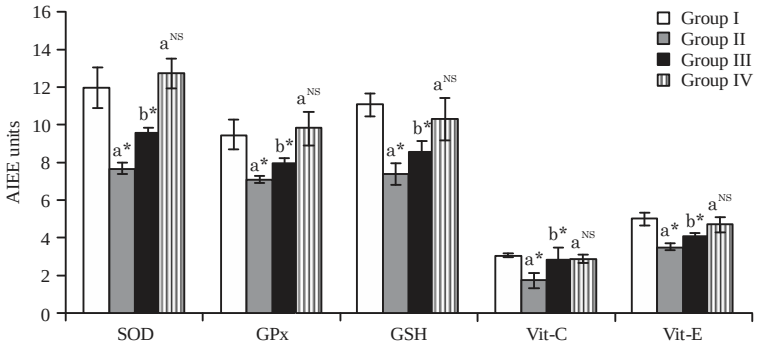

Fig. 3: Effect of AIEE on the level of antioxidant enzymes in serum of experimental rats. Each value represents Mean±SD for six animals. [a]Group 1 compared with Group 2 and 4, [b]Group 3 compared with Group 2, NS: Non significant; *p<0.001, SOD: Units/mg protein, Gpx: mg of GSH utilized/mg protein/min, GSH: mg of GSH/mg protein/min, Vitamin C: mg/g of wet tissue, Vitamin E: mg/g of wet tissue

phospholipids, free fatty acids and triglycerides were significantly reduced and total cholesterol and free fatty acids were slightly altered to near normal. There were not any remarkable changes in group 4 rats.

Effect of AIEE on carbohydrate metabolizing enzymes: The efficacy of AIEE on carbohydrate metabolizing enzymes in liver and kidney of experimental rats is shown in Table 1. The activity of hexokinase, phosphoglucoisomerase and aldolase ($p<0.001$) were significantly increased and glucose-6-phosphate and fructose-1,6-diphosphatase ($p<0.001$) levels were decreased in cancer bearing rats. All the enzymes were significantly altered with the treatment of AIEE. There was no significant change in group 4 rats.

Effect of AIEE on lipid peroxidation: Lipid peroxidation (basal, hydrogen peroxide, ascorbic acid and ferrous sulphate) found to be increased in cancer bearing rats ($p<0.001$) when compared with control. Conversely, the treatment of AIEE significantly reduces the peroxidation reaction in group 3 ($p<0.001$). However, no significant changes were observed in group 4 (Table 1).

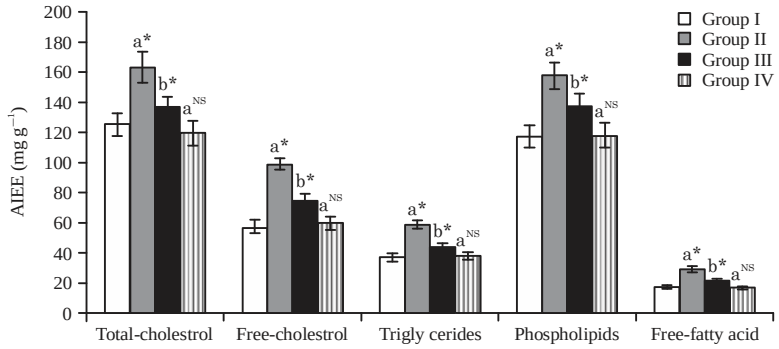

Fig. 4: Effect of AIEE on serum lipid profile in experimental animals. Each value represents Mean±SD for six animals. [a]Group 1 compared with Group 2 and 4, [b]Group 3 compared with Group 2, NS: Non significant, *p<0.001

Table 2: Effect of AIEE on the level of nucleic acids and ATPase in liver and kidney tissues of experimental rats

	Normal control		Cancer control		Cancer bearing rats±AIEE		Normal rats +AIEE	
Parameters	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Level of nucleic acids								
DNA (mg g^{-1} of wet tissue)	6.5±0.02	6.2±0.02	7.9±0.04^{a}*	7.1±0.02^{a}*	6.8±0.06^{b}*	6.6±0.12b*	6.6±0.09c^{ns}	6.15±0.08^{ans}
RNA (mg g^{-1} of wet tissue)	5.02±0.12	4.95±0.01	6.01±0.08^{a}*	5.8±0.06^{a}*	5.08±0.15^{b}**	5.12±0.12^{b}*	5.03±0.21^{cns}	5.02±0.06^{ans}
Levels of ATPases								
Na±/K$^{\pm}$- ATpase	0.42±0.08	0.72±0.06	0.29±0.09^{a}*	0.43±0.03^{a}*	0.40±0.02^{b}**	0.69±0.02^{b}*	0.39±0.02^{cns}	0.74±0.05^{ans}
Ca^{2+}-ATpase	0.37±0.01	0.49±0.03	0.18±0.09^{a}*	0.32±0.02^{a}*	0.27±0.09$^{b@}$	0.44±0.03$^{b@}$	0.35±0.09^{cns}	0.56±0.04^{ans}
Mg^{2+}-ATpase	0.30±0.01	0.54±0.04	0.25±0.01^{a}*	0.43±0.03^{a}*	0.28±0.08^{b}**	0.46±0.04^{b}*	0.31±0.01^{cns}	0.57±0.05^{ans}

Each value represents Mean±SD of six animals, a: Group 2, 4 compared with group1, b: Group 3 compared with Group 2, @: p<0.05, *p<0.001, ^{ns}: Not significant

Effect of AIEE on the nucleic acids levels: The levels of nucleic acids (DNA and RNA) in liver and kidney in cancer bearing rats were significantly increased (p<0.001). Treatment of AIEE to the cancer bearing rats were significant decrease the nucleic acid levels (p<0.001). However, no significant changes were found in group 4 when compared to the control (Table 2).

Effect of AIEE treatment in ATPase enzymes: The activities of Na^{+}/K^{+}, Ca^{2+} and Mg^{2+} ATPases in the erythrocyte membrane of control and experimental rats shown in Table 2. A highly significant decrease in Na^{+}/K^{+} and Mg^{2+} (p<0.001) and significant increase in Ca^{2+} (p<0.001) ATPases levels were obtained in cancer bearing rats when compared to the normal rats, the levels were significantly increased in Na^{+}/K^{+} and Mg^{2+} and significantly decreased Ca^{2+} ATPase (p<0.001) in AIEE treated cancer bearing rats. No difference was observed in AIEE treated control rats when compared to normal control rats (Table 2).

DISCUSSION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. The curative treatment such as tumour resection and liver transplantation are not feasible in advanced stages of HCC (Herold *et al*., 2002). Therefore, searching for effective chemotherapeutic agents is important to improve the survival rate of patients with advanced or recurrent HCC. Furthermore HCC is well known for its multidrug resistance poor response to current chemotherapeutic agents (Geng *et al*., 2003). Hence, it is necessary for the evaluation of new active drugs against HCC. In

recent years, attention has been focused on the identification of naturally occurring plant active compounds as possible chemo-preventive agents (Rajamanickam *et al*., 2014). Several studies have shown that certain naturally occurring mangrove plant viz., *Ceriops decandra*, *Heritiera fomes*, *Rhizophora apiculata* and *Excoecaria agallocha* extracts possess substantial anti-carcinogenic effects against variety of chemicals (Boopathy *et al*., 2011; Patra and Thatoi, 2013; Prabhu and Guruvayoorappan, 2012; Patil *et al*., 2011). These studies support the anticancer effect of ethanolic extract of *A. ilicifolius* against DEN induced hepato-cellular carcinoma.

The results of the present study, expressed that the food and water intake in different experimental groups were found to be unaltered. This feature is of paramount importance, because nutritional depreciation causing body weight loss may parellel a decrease in tumour volume (Waitzberg *et al*., 1989). The total body weights to the tumour bearing animals were declined when due to changes in energy metabolism during tumour formation. Results observed from the present study, indicate that in this particular two stage model of hepatocarcinogenesis in rats, the administration of AIEE greatly reduced the incidence, multiplicity and size of visible persistant nodules. Although, it is evident that not all the hepatocyte nodules become cancerous during the lifespan of the animals, numerous observations support the concept that the nodules are the precursors of hepatic cancer (Jacobs *et al*., 1981; Farber, 1984). Moreover, there is a large body of evidence to show the correlation between the number and size of hyperplastic nodules and hepatocarcinoma in both experimental and human disease (Farber, 1990). The reduction of nodules growth in extract treated animals may be due to the cytotoxic nature of the plant extract. Thus, *A. ilicifolius* had maximum effect in reducing the number and nodule growth, which were not mediated through the impairment of nutritional status in the experimental animals. The result indicates that *A. ilicifolius* possess antitumour activity.

Body weights were steadily increased in drug treated animals. This indicated that *A. ilicifolius* extract reduced the tumor incidence and the energy metabolism. In the present study the liver and kidney weight were increased in cancer bearing animals. Biochemical tumor marker enzymes are used to screen particularly tumorous conditions for differential diagnosis, prognosis, monitoring the progress and for assessing the response to therapy (Ludwig and Weinstein, 2005). These enzymes are more unique and changes in their activities reflect the effect of proliferation of cells with growth potential and its metabolic turnover. The rise in their activities is shown to be good correlation with the number of transformed cells in cancer conditions (Wulfkuhle *et al*., 2003).

Transaminase serves as index of liver injury and can be used to confirm the carcinogenic conditions. In the present investigation, the elevated levels of transaminase such as aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were observed in serum of cancer bearing animals. The altered activities of ALT and AST in serum were normalized after *A. ilicifolius* extract treatment. Alkaline phosphatase (ALP) is the membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. This enzyme activity is used as a specific tumor marker during diagnosis in the early detection of cancer (Yasuda *et al*., 2014). Alkaline phosphatase has been reported to be involved in the transport of metabolites across the cell membranes, protein synthesis, synthesis of certain enzymes, secretary activities and glycogen metabolism (Pfeiffer-Guglielmi *et al*., 2014). The ALP is also responsible for the hydrolysis of monophosphoric esters, which is essential in many biological. An elevated activity of alkaline phosphatase (ALP) is noticed in the serum and liver of the cancer bearing animals. Rise in the phosphatase activity in cancer bearing animals may be due

to the disturbance in the secretory activity or in transport of metabolites or may be due to altered synthesis of certain enzymes in cancer condition. 5-Nucleotides (5'-ND) enzyme hydrolyses nucleotides with a phosphate group on carbon atom of the ribose. It is found to be widely distributed in tumor tissue. In present investigation, an increased activity of 5'-ND was observed in liver cancer bearing animals. This elevation of the marker enzyme may be correlated with the progression of the malignancy. Walia *et al*. (1995) have reported that the higher activities of 5'-ND in cancerous liver tissue in humans. In the present study, the drug treated animals brought back this enzyme activity to near normal level indicating the antitumor property of *A. ilicifolius*. The γ-GTP is a membrane-bound enzyme that located on the surface of cells exhibiting large secretary or detoxification activities (Yao *et al*., 2000). The enzyme level is raised in serum, liver in condition like cholestasis and bile duct necrosis and is also considerable to be one of the best indicators of liver damage. A variety of substances including xenobiotic becomes γ-GTP substrate after their conjugation to GSH, occurring mainly in the liver. In the present investigation, the activity was found to increase in DEN-induced liver cancer animals. The chemo-protective nature of *A. ilicifolius* might have helped in stabilizing the cell membrane and also prevented the loss of functional integrity of the cell membrane. The LDH is a tetrameric enzyme and is recognized as a potential tumor marker in assessing the progression of the proliferating malignant cells. It is a fairly sensitive marker for solid neoplasm (Helmy *et al*., 1998). The elevated levels of LDH in cancer bearing animals may be due to the enhanced glycolysis during the growth of tumor (Nakashima *et al*., 2000). Administration of *A. ilicifolius* extract reverted the activity of LDH to near normal in drug-treated animals may be due to the inhibition of glycolysis and render the protection to the membrane. Carbohydrate moieties of glycoprotein have been implicated in the transport of metabolites across cell membranes and also a direct relation between glycoproteins and tumor genesis is observed (Deuschle *et al*., 2015). In the present study, the levels of glycoproteins hexose, hexosamine and sialic acids were significantly increased in DEN induced cancer bearing animals. This change in surface carbohydrate during cellular differentiation and neoplastic transformation suggests the importance in physiology and behavior of the cells. The increased levels of hexose and hexosamine correlated with extent of malignant disease (Patel *et al*., 1990). Elevated level of sialic acid can be useful for early detection of cancer, stating prognosis of the disease, degree of metastasis and recurrence (Shanmugam and Nagarajan, 1992). In the present investigation, the levels of hexose, hexosamine and sialic acid were increased in liver and kidney of cancer bearing animals. Administration of *A. ilicifolius* extract decreased the levels of glycoproteins in drug treated animals may be due to alteration in cell membrane glycoprotein synthesis and structure.

Hexokinase levels occupy an important place in determining the glycolytic capacity of cancer cells. A direct correlation has been observed between glycolytic activity and hexokinase in a variety of tumor cell lines. Significant increase in the activity of hexokinase was observed in cancer bearing animals. This may be due to the fact that tumors catabolize large amount of glucose. The functional significance of hexokinase is that it commits glucose metabolism (Pazarentzos and Bivona, 2014). In the present study increased levels of phosphoglucoisomerase were observed in liver and kidney which may be due to the higher glycolytic rate in liver tissues and further leakage from the destruction of neoplastic tissues. Alteration in the activity of phosphoglucoisomerase might be expected to influence the proportion of glucose-6-phosphate metabolized via the glycolytic pathway (Kaliaperumal *et al*., 2014). Aldolase, another key enzyme in the glycolytic pathway is also

increased in DEN-induced tumor conditions. The increased level of aldolase was observed in liver and kidney of the cancer bearing animals. Glucose-6-phosphatase is a marker enzyme for liver microsomal activity and is greatly inhibited in cancer bearing animals. Baggetto (1992) suggested that the decreased level of glucose-6-phosphatase reveals the progressive failure of gluconeogenesis in cancerous conditions. Generally, lactate production from glucose rises and concomitantly glucose production from pyruvate decreased during the progression of tumor growth. Thus, the observed reduction in activities of these enzymes in tumor bearing animals may be due to the higher lactate production in neoplastic tissues. Furthermore, it has been proved the tumor utilize a large proportion of lactate of glycolysis and protein synthesis. After *A. ilicifolius* extract treatment, the alterations in the levels of these enzymes were reverted back to near normal, which may be due to inhibition of glycolytic pathway and activation of gluconeogenesis. Thus, the plant extract may interrupt the energy requirement of tumor tissues and lead to the suppression of tumor growth (Sivalokanathan *et al*., 2005).

Nucleic acids play an important role during neoplastic transformation. The DNA content is found to be an independent indicator of prognosis, since the size of the tumor often correlates well with DNA content of tumor (Ellis *et al*., 1991). DNA synthesis is limited in normal hepatocytes which have a very slow rate of cell division (Board *et al*., 1990). In the present investigation, the RNA level in liver and kidney of cancer bearing animals was increased. This may be due to the increased DNA content which might have led to an increased transcription and thereby elevated RNA content in cancer condition. In the present investigation, the DNA and RNA levels were normalized in drug treated animals. Thus, administration of *A. ilicifolius* extract controlled the nucleic acid biosynthesis and exhibited the tumor inhibitory effect during treatment.

Mitochondria isolated from a wide variety of neoplastic tissues were shown to possess low efficiency for ATP production and reduced ATPases activity. The membrane bound ATPases like Na^+/K^+ ATPases, Mg^{2+} ATPases and Ca^{2+} ATPases are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP hydrolysis (Stekhoven and Bonting, 1981). Thus, it has a direct role in regulating cell volume. The activity of Na^+/K^+ ATPases can also be regulated by hormones, proteins and secondary messengers. The activities of Na^+/K^+ ATPases are highly susceptible to the lipid peroxidation, which often is generated, in cancerous condition (Selvendiran and Sakthisekaran, 2004). In the present investigation, the levels of Na^+/K^+ ATPases has found to be decreased significantly in erythrocyte membrane and in liver of the cancer bearing animals. The calcium pump of Ca^{2+} ATPases in plasma membrane because of its high calcium affinity, has been proposed as the structural responsibility for the maintenance of cytoplasmic calcium concentration at the submicromolar level (Benaim *et al*., 1993). Magnesium plays a fundamental role in the structural metabolism and bioenergetics of the cell. Further, it is required for the synthesis of protein, nucleic acid and number of mitochondrial processes. In the present investigation, the decreased level of Mg^{2+} ATPases was observed in cancer bearing animals. The increased level of LPO in the erythrocyte membrane, liver and kidney might be responsible for the decreased activity of this enzyme. Abnormal lipid peroxidation affects the membrane bound ATPases activities and their levels were decreased due to the excessive production of thiobarbituric acid reactive substance (Rauchova *et al*., 1995). Elevated cholesterol level proceeds the observed changes in DNA and protein content suggested a link between cholesterol and DNA synthesis pathway (Sreejayan and Rao, 1997). In the present study was observed the elevated levels of Total Cholesterol (TC), Free Cholesterol (FC), Phospholipids (PL), Triglycerides (TG's) and Free Fatty Acids (FFA) in the cancer bearing animals.

CONCLUSION

Mangrove plant based medicine has the prosperity of components with potential bioactivity in the treatment of cancer. However, the precise mechanism and identification of the bioactive constituent which precludes its therapeutic application needs to be understood. In the present study, it have been able to evaluate the anticancer action of *A. ilicifolius* extract in DEN induced hepato-carcinogen rats. The ethanolic extract of *A. ilicifolius* acts as an anticancer agent confirmed through carbohydrate, lipid and antioxidant metabolic enzymes in serum, liver and kidney of experimental rats. The result concludes ethanolic extract of *A. ilicifolius* has an effective chemo-preventive agent. Further isolation of active principles from *A. ilicifolius* extracts which is responsible for antioxidant and anticancer effect is needful for drug development.

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