Research Article

Effect of *Lycopersicon esculentum* (tomato) on membrane-bound ATPases against diethylnitrosamine (DEN) induced and phenobarbital (PB) promoted hepatocellular carcinoma (HCC) in rats

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Abstract

Objective: To investigate the effect of *Lycopersicon esculentum* (tomato) on membrane-bound ATPases in diethylnitrosamine (DEN) induced and phenobarbital (PB) promoted hepatocellular carcinoma (HCC) in rats. **Materials and Methods:** The hepatocarcinogenesis was initiated with a single dose in male wistar albino rats (n=6) by DEN (200 mg/kg, *i.p.*). Two weeks later 0.05% w/v of phenobarbital (PB) was administered through drinking water for 16 weeks. The treatment group rats (n=6) received a similar dosage as above along with enzymatic extract of *L. esculentum* (250 mg/kg b.w. *p.o.*) from week three onwards. The effects of *L. esculentum* on membrane-bound ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) activity in liver tissue were determined. Estimation of the serum electrolytes such as sodium (Na^+), potassium (K^+), magnesium (Mg^{++}), and calcium (Ca^{++}) were also assessed in the serum at the end of experimental period, along with morphological analysis and histopathological studies. **Results:** In our study, DEN/PB treated rats had significantly ($p < 0.001$) decreased activities of membrane-bound ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) in liver tissue. Oral administration with the enzymatic extract of *L. esculentum* to rats resulted in significant ($p < 0.001$; $p < 0.01$) reversal of these enzymes activities to near normal in liver tissue when compared with DEN/PB treated rats. It also significantly alters the levels of serum electrolytes ($p < 0.001$; $p < 0.01$) to near normal for the treatment group (*L. esculentum*) compared to DEN/PB. Treatment with the extract of *L. esculentum* normalized the histological architecture as found to be disrupted in DEN/PB group. **Conclusion:** The present study suggests that administration of *L. esculentum* has the potential to restore deranged activity of membrane-bound ATPases against DEN induced and PB promoted liver cancer.

Keywords: *Lycopersicon esculentum*, membrane-bound ATPases, hepatocellular carcinoma (HCC), Diethylnitrosamine (DEN)

Introduction

The hepatocellular carcinoma (HCC), a major health care complication of the liver cancer accounts as the second leading cause of cancer deaths among males in developing countries. The HCC, also called the most common primary malignant hepatoma (70-85% of occurrence) is responsible for a worldwide incidence of over one million cases annually. The development of HCC is frequently related to infection with hepatitis B and/or C viruses, exposure to aflatoxin, cirrhosis, environmental pollutants, iron overload, obesity, nitrosamines and diabetes mellitus (Torre et al., 2015; Das et al., 2016; Giovannucci et al., 2010).

Diethylnitrosoamine (DEN), the well-known environmental hepatocarcinogen of nitrosamine family has been widely used in experimental animal models for producing reproducible tumors. An effective hepatotoxin and hepatocarcinogen, upon administration to experimental animal causes the generation of reactive oxygen species (ROS) thereby forming DNA carcinogen adducts in the liver resulting in oxidative stress and cellular injury. It is synthesized endogenously and found in varieties of foods as cheese, salted and dried fish, soybean, processed meat, tobacco smoke, alcoholic beverages, cosmetics, pharmaceutical substances, etc. (Bartsch et al., 1989; Sivaramakrishnan et al., 2008).

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Phenobarbital (PB), the class of the barbiturates has been reported to encompass an effective tumor promoting property in the liver of animal models. The alteration of DEN initiated cells into foci followed by increase in the cytochrome P450 expression eventually augments the potency of DEN and oxidative stress making it a promoter of HCC (Pitot et al., 1987; Imaoka et al., 2004).

The ATPases (Na$^+$/K$^+$-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) are membrane-bound enzymes which are vital in maintaining the homeostasis (ionic gradients between aqueous intra and extracellular phases) of metabolic needs in the body (Ademoglu et al., 2000; Bloj et al., 1973). Hence, they regulate osmotic pressure, cellular volume and membrane permeability (Reddy and Philip, 1992). The peroxidation of membrane lipids during hepatic injury results in the alteration of structural and functional characteristics of the membrane, thereby affecting the activities of the membrane-bound ATPases (Kannampalli et al., 2007). The hepatic carcinogenesis results in the changes for the levels of electrolytes and cell viability by altering the membrane integrity.

Lycopersicon esculentum Mill. (Tomato) belongs to the family Solanaceace and is one the most important cultivated "protective foods" due to its special nutritive value (Sakarkar et al., 2011; Campbell et al., 2007). The plant exhibit various activities including treatment of chronic diseases as cardiovascular (Bhowmik et al., 2012) and certain types of cancer (Nkondjock et al., 2005; Huang et al., 2008; Salman et al., 2007). It is widely used for its anti-diabetic (Ali and Agha, 2009), anti-ageing (Bhowmik et al., 2012; Ganesan et al., 2012), anti-oxidant (Bose and Agrawal, 2007), anti-obesity (Bhowmik et al., 2012) and anti-fertility properties (Gupta and Kumar, 2002). In our previous study, we have reported that *L. esculentum* possess chemopreventive activity against DEN-induced and PB-promoted liver cancer (Das et al., 2016). In the present study, we examined the effects of *L. esculentum* on membrane-bound ATPases in diethylnitrosamine (DEN) induced HCC.

Materials and methods

Plant material collection and enzymatic extraction

The fruits of *Lycopersicon esculentum* Mill. were bought locally and was authenticated by Dr. Arun Kumar B. Sonappanavar, Associate Professor, Department of Botany, P.C. Jabin Science College, Vidyanagar, Hubballi, Karnataka.

The fresh tomatoes were first washed with tap water to remove the external dirt. The tomato peels were subjected to two-step extraction process as suggested by Lavecchia and Zuorro with slight modification. The peels of the tomato in the first step were allowed to treat with enzymes i.e. cellulase (1.5%) and pectinase (2%) in citrate buffer at 25ºC for 4 h, followed by extraction in the second step carried out by tri-mixture of solvent as Hexane: acetone: ethanol (2:1:1) at 40ºC for 3 hour in a soxhlet extractor (Lavecchia and Zuorro, 2008; Ranveer et al., 2013). The enzymatic extract was concentrated under reduced pressure and stored in desiccators until further use.

Chemicals and reagents

Diethylnitrosamine (DEN), cellulase and pectinase of *Aspergillus niger* were purchased from Sigma Aldrich Chemical Company (USA). The adenosine triphosphate (ATP) was purchased from Hi-Media Pvt. Ltd. (Mumbai, India). Other chemicals (solvents and reagents) used in this study were of analytical grade obtained from S.D. Fine Chemicals (Mumbai, India).

Ethical consideration

Male albino rats of Wistar strain weighing about 150-200g were used for the study. The animals were acclimatized for one week under standard laboratory conditions by housing them in polypropylene cages (six animals per cage) maintained at 27 ± 2ºC under 12 h dark and light cycle. Animals were fed with standard rat feed (Gold Mohur Lipton India Ltd.) and water was provided *ad libitum*. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol prior to the beginning of the project work (Ref. No. 01-IAEC.HBL-29/2014).

Experimental design

The rats were randomly divided into three groups, each group containing six animals as depicted in table 1 (Pradeep et al., 2010; Wang et al., 2010). At the end of experimental protocol (16 weeks), the rats were fasted for 12 h, anesthetized under mild ether anaesthesia and blood was collected in heparinized tubes for the separation of serum. The liver tissue was isolated, washed with saline, blotted of blood and macroscopically observed for visible liver tumors or nodules on the liver surface and was recorded. One portion of the liver was taken for preparation of 10 % w/v homogenate in 0.15 M Tris-HCl buffer (pH 7.4) and was used for the assay of membrane-bound ATPases. The serum electrolytes were estimated using commercially available kits using semi-auto analyzer (ERBA Mannheim Chem-5 Plus v2).

Table 1. Experimental design and treatment protocol

Groups	Status	Treatment
I	Vehicle control	0.9 % w/v normal saline daily (*p.o.*) for 16 weeks.
II	Carcinogenic control (DEN + PB)	The DEN at a single dose of 200 mg/kg (body weight, *i.p.*) on 1st week followed by administration of Phenobarbital (0.05% w/v) daily after 2nd week through drinking water for up to 16 weeks.
III	Positive control DEN+PB+*L.E* (250)	The positive control group was allocated with the same experimental design as mentioned in Group II along with the enzymatic extract of *L. esculentum* after 2nd week (5 days/week, *p.o.*) at a dose of 250 mg/kg (body weight) for up to 16 weeks.

Estimation of membrane-bound ATPases

The Na^+K^+-ATPase activity was assayed according to the method described by Bonting, 1970. Briefly, 1.0 ml of Tris-HCl buffer and 0.2 ml each of $MgSO_4$, NaCl, KCl, EDTA, ATP were added to test tube containing 0.2 ml of homogenate. The mixture was then incubated at 37ºC for 15 min. The reaction was arrested by addition of 1 ml of 10% TCA, later mixed well and centrifuged. Mg^{2+}-ATPase activity was assayed according to the method described by Ohnishi et al. (1982). The incubation mixture containing 0.1 ml each of Tris-HCl buffer, $MgCl_2$, ATP and homogenate in a test tube was incubated at 37ºC for 15 min. The reaction was arrested by addition of 1 ml 10% TCA, was mixed well and centrifuged. Ca^{2+}-ATPase activity was assayed according to the method described by Hjerten and Pan, (1983). As described, the incubation mixture containing 0.1 ml each of Tris-HCl buffer, $CaCl_2$, ATP and homogenate in a test tube was incubated at 37ºC for 15 min. The reaction was arrested by addition of 1 ml of 10% TCA, was mixed well and centrifuged. All these enzyme activities were expressed as µmoles of inorganic phosphorus liberated per mg protein per minute as described in the section determination of inorganic phosphorus (Pi).

Determination of inorganic phosphorus (Pi)

The phosphorus content of the supernatant was estimated by the method described by Fiske and Subbarow, (1925). Briefly, 1.0 ml of supernatant was taken and the volume was made up to 5.0 ml with distilled water. To this, 1.0 ml of 2.5% of ammonium molybdate and 0.5 ml of ANSA (1-amino-2-naphthol-4-sulphonic acid) were added. The colour developed in 20 min was read using water as blank at 620 nm. A standard graph was prepared taking different concentrations of Phosphorus of 16 to 80µg/ml. The values were finally expressed as µM of inorganic phosphorus liberated/mg protein/min.

Preparation of paraffin sections (H and E stain)

A portion of the liver for each animal was fixed in 10% formalin and the tissue was dehydrated in ascending series of alcohol followed by clearing with xylene. The tissue was impregnated with paraffin for overnight and immediately sections of 5µ thick were cut from each block. The sections were stained with H&E to study the general histological architecture.

Statistical analysis

Statistical analysis was analyzed by using one-way ANOVA followed by Bonferroni's multiple comparison test. Results were expressed as mean ± standard error mean (S.E.M) from six rats in each group. Graph Pad Prism Software version 6.0 was used for the statistical purpose and for drawing the bar charts.

Results

Body, liver and relative liver mass

The changes in mean body mass in rats was observed during the period of experiment (16 weeks) in the control and treated groups as illustrated in table 2. The DEN/PB treated group showed significant decrease in mean body mass when compared with the normal group. The treatment group (DEN/PB + *L.E*) showed significant increase in mean body mass when compared to DEN/PB group.

Table 2. Effect of *L. esculentum* on body, liver and relative liver mass of normal and experimental animals

Groups	Initial weight (g)	Final weight (g)	Liver weight (g)	Relative liver weight (g %)
I	155.2 ± 1.07	262.3 ± 1.90	8.52 ± 0.20	3.65 ± 0.24
II	152.7 ± 1.35	202.5 ± 3.22	9.98 ± 0.30a**	4.74 ± 0.18a*
III	153.7 ± 1.25	218.3 ± 3.56	9.02 ± 0.22b*	3.82 ± 0.24b*

Values are expressed as Mean ± S.E.M. (n=6). Comparisons are made between: a-Normal rats (Group I); b-DEN/PB treated rats (Group II). **$p< 0.01$; *$p< 0.05$.
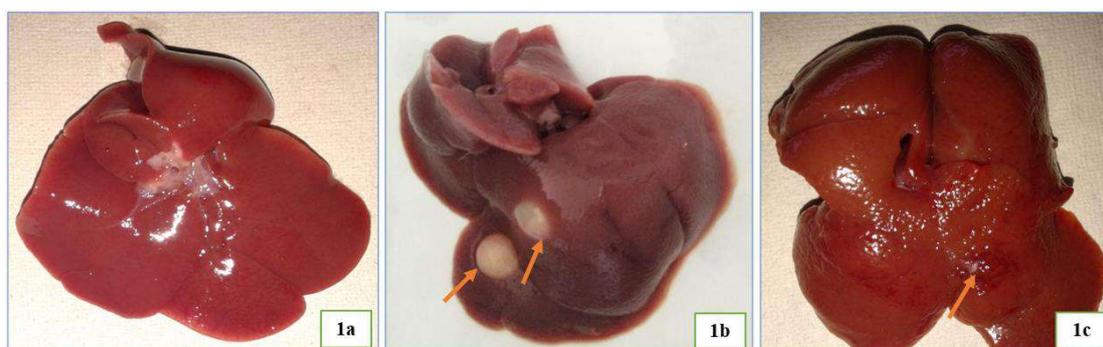

Figure 1. Macroscopic gross liver tumors or nodules at the end of experimental period (16 week) of normal and experimental group showing: a) A control liver with normal morphology and without any nodules. b) DEN/PB treated liver with gradual enlargement and whitish nodules. c) The treatment with *L. esculentum* show decreased sign of tumor development in the rat liver. Arrows indicate nodule formation.

The DEN/PB treated group showed significant increase ($p< 0.01$) in liver and relative liver mass when compared to normal group. The treatment group (DEN/PB + $L.E$) showed significant decrease ($p< 0.05$) in liver and relative liver mass when compared with DEN/PB group (table 2).

Morphometric analysis of the liver

The gross liver tumors or nodules analysis of the liver in DEN/PB and DEN/PB + L.E treated groups at week 16 are represented in figure 1. The morphometric analysis of the normal control liver reveals normal morphology of the liver without any presence of subcapsular nodules throughout the 16-week experimental period. On the conflicting, the macroscopic gross appearance in the DEN/PB treated group showed several whitish nodules and foci on the surface of the liver. Oral administration of the enzymatic extract of *L. esculentum* resulted in marked inhibition of tumor development in the rats. The average number of nodules per nodule-bearing liver was significantly lower when compared with DEN/PB group (figure 2).

Estimation of membrane-bound ATPases

The status of membrane-bound ATPases of normal and treated animals is depicted in figure 3. Animals administered with DEN/PB showed significant decreased ($p< 0.001$) activities of total ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) in liver tissue when compared with normal group. The treatment group (DEN/PB + $L.E$) significantly ($p< 0.001$; $p< 0.01$) overcame the deleterious effects when compared with DEN/PB group.

Estimation of serum electrolytes

The DEN/PB group showed significant decreased ($p<0.001$) level of serum electrolytes (Na^+, K^+, Mg^{++}, and Ca^{++}) compared to normal group. There was a significant increase ($p< 0.001$; $p< 0.01$) in electrolytes level in the treatment group (*L. esculentum*) compared to DEN/PB group as depicted in figure 4.

Liver histopathological analysis (Hematoxylin and eosin)

A representative view of the liver sections stained with H & E are shown in figure 5. The normal group animals (figure 5a) showed no sign of necrosis. Hepatocytes structure looks normal with central vein and sinusoids and there were no pathological changes. In DEN/PB treated group (figure 5b), there were signs of centrilobular degeneration, sinusoidal congestion, and spotty necrosis. Portal triad degeneration and ballooning hepatocytes were also observed. In *L. esculentum* treated group (figure 5c), there were sign of decreased portal triaditis, along with central vein and sinusoidal congestion.
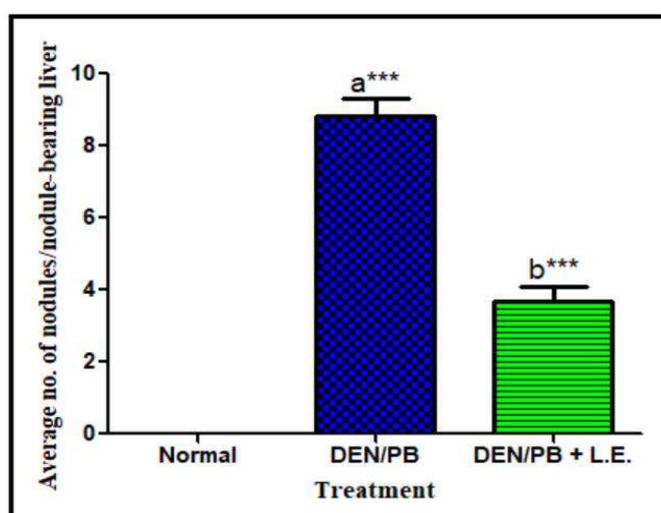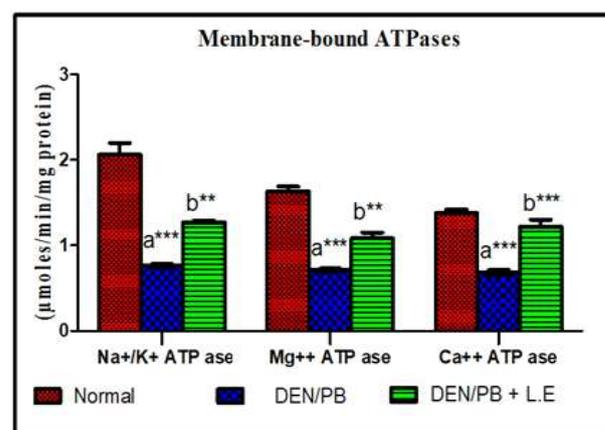

Figure 3. Effect of *L. esculentum* on total ATPases of normal and experimental animals. Values are expressed as Mean ± S.E.M. (n=6). Comparisons are made between: a-Normal rats (Group I); b-DEN/PB treated rats (Group II). **$p< 0.01$, ***$p< 0.001$.

Figure 2. Effect of *L. esculentum* on liver tumor and average no. of nodules per nodule-bearing liver of normal and experimental animals. Values are expressed as Mean ± S.E.M. (n=6). Comparisons are made between: a-Normal rats (Group I); b-DEN/PB treated rats (Group II). ***$p< 0.001$.
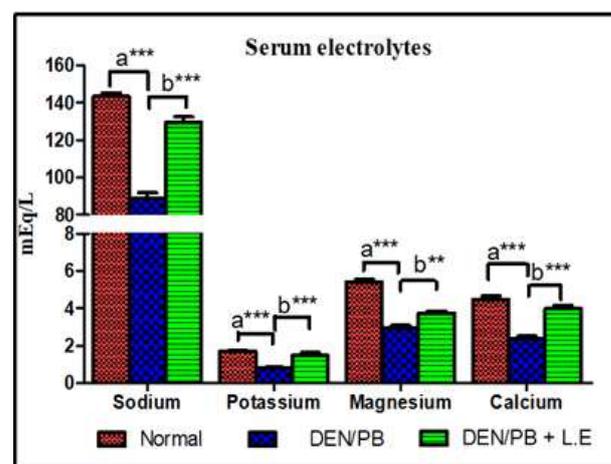

Figure 4. Effect of *L. esculentum* on serum electrolytes of normal and experimental animals. Values are expressed as Mean ± S.E.M. (n=6). Comparisons are made between: a-Normal rats (Group I); b-DEN/PB treated rats (Group II). **$p< 0.01$, ***$p< 0.001$.
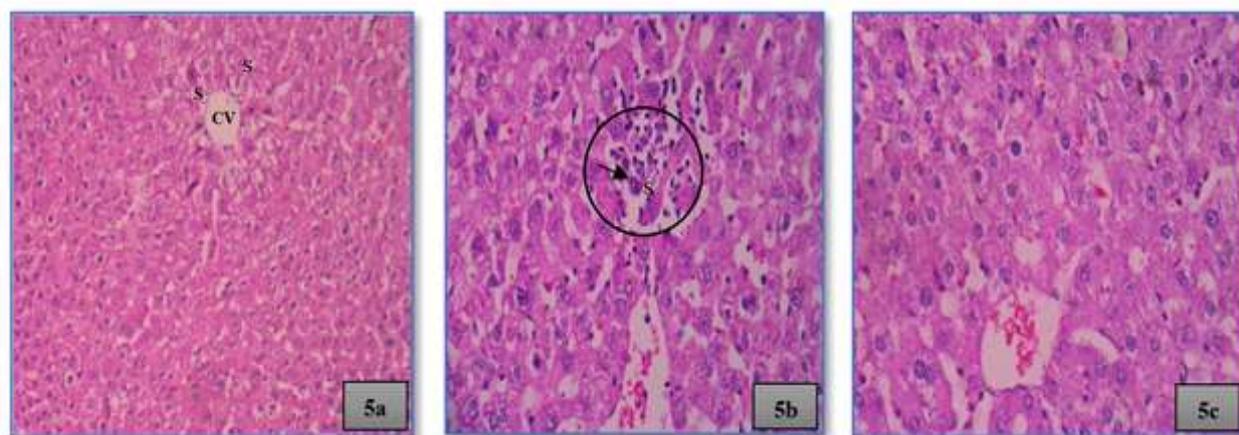
Figure 5. Photomicrographs of liver sections of normal and experimental group showing: a) A normal liver architecture showing classic hepatic lobule containing central vein (CV) with blood sinusoids (S) in between. b) A disturbed liver architecture apparently showing dilated and congested blood sinusoids (S) with centrilobular degeneration (encircled) and spotty necrosis (arrow) in DEN/PB treated group. c) The treatment with *L. esculentum* showed decreased sign of central vein and sinusoidal congestion (a × 20, b & c ×40).

Discussion

In the present investigation, it was observed that the decrease in the activities of total ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) in liver tissue of DEN/PB induced HCC animals may be due to increased production of free radicals leading to cell injury. The membrane-bound ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase) are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP by hydrolysis (Schuurmans and Bonting, 1981). The decrease in the activity of membrane-bound ATPases indicates the pathological changes of the membrane. Previous studies have shown that hepatic injury resulting from peroxidation of membrane lipids results in alteration of structural and functional characteristics of the membrane, thereby affecting the activities of the membrane-bound ATPases. These enzymes are susceptible to hydroperoxides and superoxide radicals, which might be the reason for their decreased activities during diethylnitrosamine administration (Kannampalli et al., 2007). The damage of plasma membrane occurs directly through interaction with the membrane components such as the ion-dependent ATPases and ion channels and indirectly because of cytosolic damage. The inhibitory function of ion-dependent ATPases leads to disturbances in ion homeostasis resulting in impaired signal transduction and altered cellular metabolism, which further may cause alteration in cell membrane permeability, integrity and disturbances of vital function respectively.

Administration with the enzymatic extract of *L. esculentum* to DEN/PB induced HCC rats significantly elevated the activity of total ATPase, Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase in liver tissues when compared with that of the DEN/PB control rats. The restoration of membrane-bound ATPase activity in DEN/PB induced HCC rats to near normal values could be due to limiting the degree and levels of oxidation due to an increase in the reduced glutathione (GSH) and lipid peroxidation content of the liver (Jamshidzadeh et al., 2008; Weremfo et al., 2011).

In the present study, administration of DEN/PB significantly decreased the serum electrolytes levels of sodium (Na^+), potassium (K^+), magnesium (Mg^{++}), and calcium (Ca^{++}) respectively. Several studies have claimed similar decrease in the levels of serum electrolytes upon DEN/PB administration. The most possible cause for depleted serum sodium in DEN/PB induced group is due to retention of excess water (Nakano et al., 1996). Potassium is the principal cation of intracellular fluid and plays an important role in the maintenance of acid-base balance. Several disorders of potassium metabolism and significant decrease in serum calcium have been described in association with liver diseases (Perez et al., 1983; Rocchi et al., 1994; Sullivan et al., 1979). Magnesium, the most essential micronutrients plays a vital role in maintaining the immune system and studies claiming a significant decrease in serum magnesium level has been reported in liver cirrhosis patients (Tam et al., 2003). Also, ascites plays a significant role in the reduction of serum electrolytes in liver cirrhosis (Papadakis and Arieff, 1988). The altered levels of the serum electrolytes reversed to nearly normal level after the rats were treated with the extract of *L. esculentum*. It suggests that the alterations of essential electrolytes might play a role in DEN/PB induced liver injury in rats.

The changes in the parameters (membrane-bound ATPases and serum electrolytes) after treatment with DEN/PB were reaffirmed by histopathological observations in rat liver. In

the current study, liver tissue section of DEN/PB group showed significant pathological conditions such as focal haemorrhage, inflammation, ballooning hepatocyte, centrilobular degeneration, spotty necrosis, central vein and sinusoidal congestion at higher rate compared to normal and *L. esculentum* treated group. Whereas *L. esculentum* treated liver sections showed, reduced central vein and sinusoidal congestion, along with absence of ballooning hepatocyte, spotty necrosis when compared to DEN/PB suggesting its protective effect against carcinogenesis.

Conclusion

The results of this study reflect that *L. esculentum* can modulate ATPases and reduce free radical formation in DEN-induced liver cancer in albino rats. Hence, with the administration of *L. esculentum* to DEN/PB rats, it has the probability to minimize the deleterious effects of free radicals and can protect the structural integrity intimating efficacy of this agent against membrane damage tumorigenesis respectively. Further detailed investigation is necessary to discover the mechanism of action and establish its therapeutic potential in the treatment of liver cancer.

Acknowledgement

Conflict of interest statement

The authors of this publication declared that there is no conflict of interest.

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