ORIGINAL ARTICLE

Ameliorative Activity of a Green Extract of Spinach (*Spinacia oleracea*) on Paracetamol Induced Acute Liver Injury

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ABSTRACT

Context: Paracetamol overdose or toxicity often leads to acute liver injury. Spinach is a popular and traditionally revered green leafy vegetable. Functional properties of spinach (Spinacia oleracea) may be beneficial for treating this condition. **Aims:** To find out the effect of an aqueous extract of spinach in paracetamol induced liver injury. **Methods and Material:** Sixteen Swiss albino mice were randomly divided into four groups. Group-I served as control. Acute liver injury was induced in Group-II, III and IV with paracetamol. Group-III and IV were then treated with a green extract of spinach and silymarin respectively. **Statistical Analysis Used:** Analysis of Variance (ANOVA) and Post-hoc analysis was performed. **Results:** Upon induction with paracetamol the serum levels of bilirubin, hepatic transaminases, total protein and lipid peroxidation in the liver were elevated. Superoxide dismutase and reduced glutathione levels were decreased in the liver. All these parameters were reversed significantly ($p<0.05$) in Group-III comparable to Group-I and IV. **Conclusions:** This indicates that the green extract of spinach may be useful in the treatment of paracetamol induced acute liver injury.

Keywords: Acetaminophen, Bilirubin, Lipid peroxidation, Glutathione, Superoxide dismutase

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INTRODUCTION

Drug induced liver injury (DILI) is an adverse event observed in clinical practice. Since liver metabolizes a wide range of compounds including herbs and alternative medicines, the toxic by-products or end-products can cause liver damage. Paracetamol is one of the most frequently used drugs in the clinical setting. Paracetamol or acetaminophen overdose is the commonest cause of acute hepatic injury and failure resulting in death or need for emergency hepatic transplant. It is more prevalent in USA, Europe, Australia and comparatively less in Asia. It is a preventable public health concern and requires attention by both clinicians and patients. [1,2,3] Reactive Oxygen Species (ROS) play important role in the pathophysiology of DILI.Drugs and their metabolites can bind to mitochondria covalently and cause hepatotoxicity by mitochondrial dysfunction, accumulation of ROS, oxidative stress, endoplasmic reticulum stress resulting in cell death.[4] Targeting such factors is the need of the contemporary biology and medicine.

Medicinal plants are used as antidote in human diseases from ancient time period. Among the wide range of plants and

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herbs available in India, the green leafy vegetables are well recognized for their significant health-promoting properties. They contain various nutrients and chemical compounds which have functional properties. Spinach (*Spinacia oleracea*) is one of the most widely consumed green leafy vegetables. It is commonly known as *"Palak"* in India and is popular for its high nutritional value. It has a slightly pungent or sweet taste. It is a good source of retinol, beta-carotene, zeaxanthin, lutein, ascorbic acid, alpha-tocopherol, B-complex vitamins, folic acid, phylloquinone, calcium, magnesium, phosphorus, iron, selenium, zinc, omega-3 fatty acids, dietary fibre and various polyphenolic compounds like quercetin, luteolin, kaempferol etc.[5] Increased consumption of fresh vegetables that are high in polyphenols has been associated with a reduced risk of oxidative stress-induced disease. Spinach also possesses antioxidant, anti-inflammatory, anti-carcinogenic, anti-obesity, hypoglycemic, lipid-lowering and hepatoprotective properties.[6] Traditionally it is considered to be *Sheeta* (coolant), *Shleshmala* (may increase Kapha), *Bhedini* (laxative effect) and useful in various conditions including hepatic inflammation and jaundice.[7] Many scientific studies have proven the beneficial role of spinach in various liver diseases till to date.[8,9,10,1,12] But the impact of spinach on drug induced acute liver injury is not well evaluated. Thus the objective of the current study is to analyze the hepatoprotective effect of a green extract of spinach in paracetamol induced acute liver injury and hyperbilirubinemia.

MATERIALS AND METHODS

Chemical and Reagents

Spinach was collected from market and was verified by Department of Botany, VU. Paracetamol was purchased from Merck and used without further purification. Silymarin was obtained from Zydus cadila. For total bilirubin, test kit of Autospan Liquid Gold Surat, Gujrat was used and Biochemical Test kits were used for liver biomarkers from Elba science.

Plant Extract Preparation

250 gm spinach was washed thoroughly with distilled water. These leaves were dried, grinded in an electric grinder to get a powder from and sprayed over tray to avoid fungus. These leaves were subsequently boiled for 8 minutes in 100 ml of water and the extract was filtrated and stored at 4º C overnight[13].

Sample Size Calculation

Based on the study objectives, study design and available resources the following formula was used to calculate the number of animals required in each of the experimental groups,

Number of animals in each group (n) = (10/k) + 1

(k= number of experimental groups)[13]

Since we had four experimental groups,

Calculated sample size per group (n) = (10/4) + 1 = 3.5 H" 4

Animals

Swiss albino mice (20-25 days old) weighing (25-28) gm were obtained from a CPCSEA approved animal house (Registration No. 50/CPCSEA/1999) and randomly divided into four groups of four mice (n=4) each of which received standard laboratory diet (Hindustan Lever, Kolkata) and water *ad libitum*. Apart from standard food during the treatment period therapeutic food is also given to all groups. The animals were housed in large, clean, polypropylene cages in a temperature-controlled room (20±2ºC) with relative humidity (45–60%) under light and dark cycles (12 hour/ 12 hour) during the whole studyperiod.

Prior to experimentation acclimatization was done for seven days. The animals were maintained according to the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. AEC/PHARM/1503/03/2015 dated 30.11.15).

The experimental protocol is mentioned in Table 1.

Biochemical Analysis

Activities (Alanine Transaminase (ALT), Aspartate Transaminase (AST) and Alkaline Phosphatase) (For biochemical studies, blood samples were collected just before sacrifice in sterile tubes from retro-orbital plexus and allowed to clot for 45 minutes. Serum was separated by centrifugation at 6000 rpm for 15 minutes. All serum samples were sterile and hemolysis-free. Liver injury was assessed by the estimation of bilirubin (expressed as mg/dL) and liver enzyme ALP) expressed as IU/L. Total protein concentration was estimated and expressed as gm/dL.[14]

Superoxide Dismutase (SOD): 1 mM diethylenetria-minepenta acetic acid adjourned with 3 ml tris buffer,45 ml Pyrogallol mixture aliquot of assay mixture and blend and eminent optical density(OD) at 420 nm. SOD activity was indicated as units/milligram protein.

Glutathione (GSH): 0.1 ml Supernatant of tissue conciliate was attached with 2.9 ml phosphate buffer. After conciliation and then this mixture was transmitted in cuvette used as a control sample. In a cuvette 0.1 ml Supernatant of tissue homogenate also added with 1.9 ml phosphate buffer. Catalase was calculated by using this formula, AA/min×1000×3/40x mg Protein in sample(u/mg Protein).

Table 1: Experimental protocol

Groups (n=4)	Days of Treatment	Remarks	
	8th-14th day	15th-21st day	
	Induction dose	Treatment dose	
I	Nil	Nil	Control
II	Paracetamol(1ml/kg body weight)	Nil	Acute Liver Injury
III	Paracetamol(1ml/kg body weight)	Spinach Extract(3 ml/kg body weight)	Herb Treated
IV	Paracetamol (1ml/kg body weight)	Silymarin (3ml/kg body weight)	Clinical Treated

Lipid Peroxidation (LPO): Thiobarbituric acid and trichloro acetic acid were added respectively. The tissue supernatant distilled water added with this mixture then boiled on a water 95ºc for 20 minutes. Solution get pink colour after few minutes later and cooled under tap water.[15]

Histopathological Studies

For histopathological studies liver was cut off after blood collection, cleaned and dried with tissue paper. It was weighed and fixed in neutral formalin solution (10%), dehydrated in graduated ethanol (50-100%), cleared in xylene and embedded in paraffin. Stained with Hematoxylin and Eosin (H&E) dye and examined for histopathological changes under the microscope.[16]

STATISTICAL ANALYSIS

Data analysis was performed using MS-Excel (2007) and Statistical Package for Social Sciences (SPSS) version 16.0. All the data were expressed as Mean ± Standard Deviation (SD). One-way Analysis of Variance (ANOVA) was performed followed by Post-hoc analysis with the level of significancep<0.05.

RESULTS

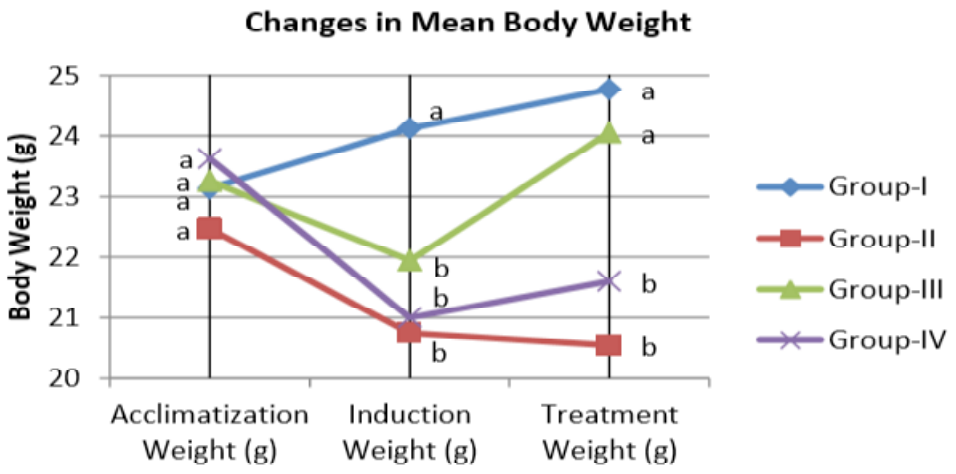
Body Weight Changes

This study reveals that at the time of acclimatization period the body weight of the mice increased and they stayed fresh but due to the application of paracetamol their weight was reduced and they became languorous. On the treatment period they gradually improved. The trend of body weight change is shown in Figure 1.

Effect of Herb on Bilirubin, ALT, AST, ALP and Total Protein

The current study showed that upon induction with paracetamol the levels of bilirubin, ALT, AST and ALP

Figure 1: Changes in Mean Body Weight of Mice During Study Period



Note: Values are expressed as Mean. Values (for the same Weight Category) with different alphabetic legends are significantly different (p<0.05). Group-I: Control, Group-II: Acute Liver Injury, Group-III: Herb Treated, Group-IV: Clinical Treated.

significantly increased from the control. But upon treatment with aqueous green extract of spinach and Silymarin the values were reversed. Similarly in case of total protein the values increased upon induction with paracetamol and reversed by herb extract and Silymarin. The values are shown in Table 2.

Effect of Herbs on Oxidative Stress

Upon induction with paracetamol the level of LPO increased whereas the levels of SOD and GSH decreased significantly. All these parameters were reversed efficiently by green extract of spinach and Silymarin the details of which are given in Figure 2.

Organ Analysis

The weight of the liver increased upon induction with paracetamol which was then reduced by theherb extract and Silymarin significantly. The details are shown in Table 5.

Histopathological Details

An extensive loss of hepatic architecture was observed upon induction with paracetamol along with vacuolation of hepatocytes. These factors were reversed in the herb treated group to near normalcy. The histological sections are shown in Figure 3.

Discussion

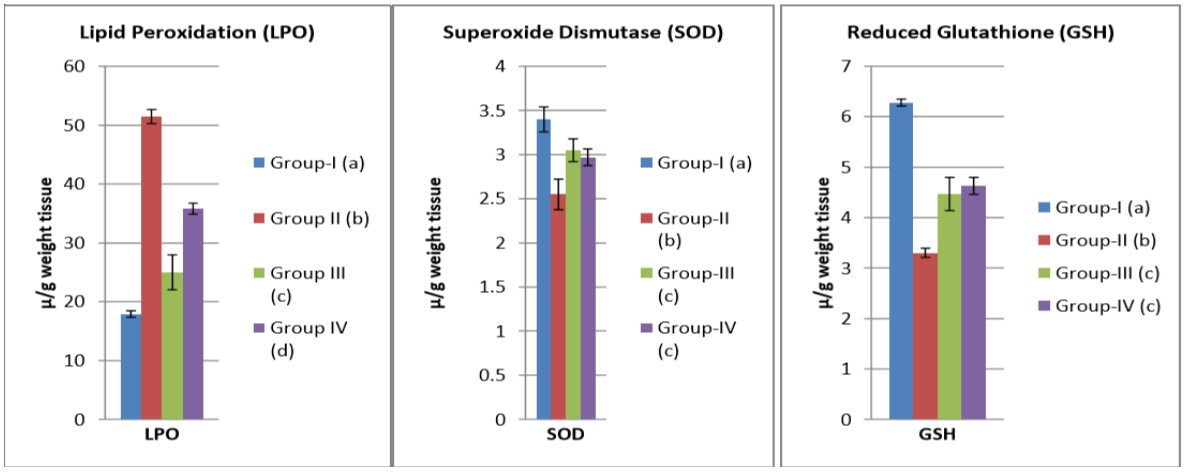
The results of our present study show that the aqueous green extract of spinach can efficiently reverse the biomarkers of paracetamol induced acute liver injury in mice. Liver is an important organ involved in the metabolism of nutrients, drugs and various other chemical compounds. In liver diseases and injury the levels of hepatic transaminases (AST, ALTand ALP) increase in the blood as a result of their release from hepatocytes due to cell damage. The regeneration and

Table 2: Effect of herb on Bilirubin, ALT, AST, ALP and Total Protein in paracetamol treated mice

Parameter	Group-I	Group-II	Group-III	Group-IV
Bilirubin (mg/dL)	0.29 ± 0.012^{a}	0.85 ± 0.026^{b}	0.31 ± 0.189^{ac}	0.33±0.01^{c}
SGPT/ALT (IU/L)	22.37±0.75^{a}	90.25±2.629^{b}	38.5±2.081^{c}	47.25±0.957^{d}
SGOT/AST (IU/L)	21.37±1.25^{a}	105.95±3.446^{b}	42.25±4.5^{c}	49.00±2.581^{d}
ALP (IU/L)	119.42±2.142^{a}	172.25±5.56^{b}	122.5±1.732^{a}	155±1.414^{c}
Total Protein (g/dL)	4.17±0.05^{a}	5.6±0.141^{b}	4.42±0.262^{a}	5.65±0.866^{b}

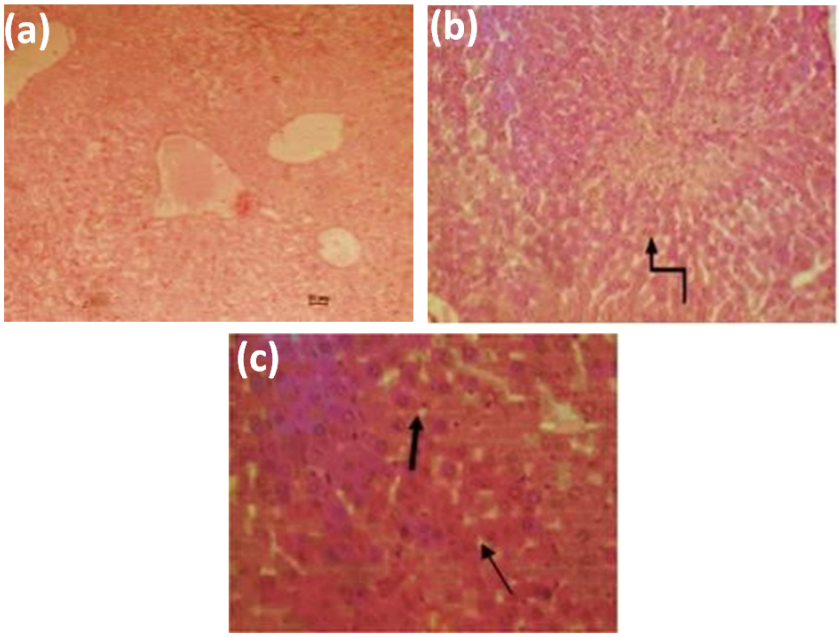
Note: Values are expressed as means ± SD (n=4). Values (for the same parameter) with different superscript are significantly different (p<0.05). Group-I: Control, Group-II: Acute Liver Injury, Group-III: Herb Treated, Group-IV: Clinical Treated.

Figure 2: Effect of herb on LPO, SOD and GSH in paracetamol treated mice



Note: Values are expressed as means ± SD (n=4). Values (for the same parameter) with different superscript are significantly different (p<0.05). Group-I: Control, Group-II: Acute Liver Injury, Group-III: Herb Treated, Group-IV: Clinical Treated.

Figure 3. Histological Sections of Liver



Note: Group-I (Control), Liver tissue showing intact endothelial layer with normal hepatic architecture and central vein; B: Group-II (Acute Liver Injury), an extensive loss of hepatic architecture (arrow) with vacuolation of hepatocytes; C: Group-III (Herb Treated), Hepatocytes with mild cytoplasmic vacuolation.

Table 5: Weight of the liver

Group	Weight of the Liver (gm)
Control	0.7 ± 0.081^{a}
Paracetamol treated	1.35 ± 0.129^{b}
Herbs treated	0.9 ± 0.081^{ac}
Silymarin	1 ± 0.081^{c}

Note: Values are expressed as means ± SD (n=4). Values with different superscript are significantly different ($p<0.05$). Group-I: Control, Group-II: Acute Liver Injury, Group-III: Herb Treated, Group-IV: Clinical Treated.

healing of hepatic tissue is further associated with a reduction in transaminase levels to normal values.[17]

Previously hydroalcoholic extract of spinach (HES) is found to be beneficial in the prevention and treatment of high-fat diet induced non-alcoholic fatty liver in rat models. In the prevention phase HES significantly reduced AST and ALP levels whereas in the treatment phase there was significant reduction in AST, ALT and ALP levels.[10]The presentstudy is the first one to evaluate the effect of aqueous extract of spinach on paracetamol induced liver injury. It was more efficient than Silymarin in reducing AST, ALT, ALP and Total Protein altered dueto paracetamol overdose. Hyperbilirubinemia was also observed in the Group-II and it was corrected in Group-III by the green extract of spinach comparable to the control and silymarin. This observation makes our study unique.

The mechanism of liver damage by paracetamol has been studied extensively. Paracetamol is metabolized in the liver and majorly converted into non-toxic glucuronate or sulfate conjugates. Later on these are secreted in urine. A minor amount of paracetamol can be metabolized via the cytochrome P450 system to form a toxic intermediate called N-acetyl-p-benzoquinoneimine (NAPQI). This compound gets conjugated to reduced glutathione, gets detoxified and secreted. If this pathway of metabolism is increased or level of reduced

glutathione is decreased toxicity sets in.[2]The critical mediators involved in paracetamol induced cell death are Reactive Oxygen Species (ROS) and peroxynitrite. NAPQI binds to certain mitochondrial proteins and leads to oxidative stress which is further boosted by c-jun N-terminal kinase (JNK) activation. Oxidative stress in mitochondria opens up Mitochondrial Permeability Transition Pore (MPTP) resulting in cessation of ATP formation, nuclear DNA fragmentation and eventually necrotic cell death.Due to this Damage Associated Molecular Patterns (DMAPs) are released leading to neutrophil infiltration. This may cause further ROS generation outside the hepatocytes.[18]All these steps can act as important targets for nutraceutical development. From our study it can be observed that the level of lipid peroxidation (LPO) was increased along with a decrease in reduced glutathione (GSH) and superoxide dismutase (SOD) in the paracetamol induced Group-II. The green extract of spinach reduced LPO levels better than Silymarin and improved the GSH and SOD levels at par with Silymarin. Spinach is a source of a variety of antioxidant and anti-inflammatory compounds which help to prevent oxidative stress and damage due to ROS production. Sang-Heui Ko *et al* found that the Total Phenolic Content (TPC) of water extract and ethanolic extract of spinach were 1.5±0.0 mg Gallic Acid Equivalent (GAE)/g and 0.5±0.0 mg GAE/g respectively. The Oxygen Radical Absorbance Capacity (ORAC) values of these extracts were also increased in a concentration dependent manner.[19] Such evidences prove the significant total phenolic content and antioxidant activity of spinach extract. We also observed an increase in liver weight, loss of hepatic architecture and vacuolation of hepatocytes in the paracetamol induced Group-II which was reversed in Group-III significantly.

CONCLUSION

Functional foods and nutraceutical development are one of the most popular areas of research in the current era. The present study highlighted the potent hepatoprotective properties of aqueous green extract of spinach(*Spinacia oleracea*) in paracetamol induced liver injury and hyperbilirubinemia. Results were comparable to the conventional drug Silymarin. The rich polyphenolic content and antioxidant properties of spinach contribute to such effects. Theseevidences show the functional properties of spinach. However further studies are required to evaluate the additional role of spinach in liver diseases.

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SOURCE(S) OF SUPPORT

Nil

PRESENTATION AT A MEETING

Nil

CONFLICTING INTEREST (IF PRESENT, GIVE MORE DETAILS)

Nil

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