Original Article

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Pharmacology Section

Effect of *Azadirachta indica* against Sodium Benzoate Induced Hepatorenal Toxicity in Wistar Rats- An Experimental Interventional Study

DEEPA KAMESWARI[1], VIGNESH EZHIL[2], VARADHARAJA PERUMAL[3], M MANJU[4], M MEGANATHAN[5], K MADHANAGOPAL[6]

ABSTRACT

Introduction: *Azadirachta indica* (Neem) is one of the widely used plants which has various medicinal properties like antipyretic, antimicrobial, antitumour, anti-inflammatory, antiulcer and antidiabetic effects. Silymarin-a milk thistle derivative has its own clinical significance. Sodium Benzoate (SB) is a versatile food preservative used in packaged food and drink industries which is consumed by the people unknowingly above World Health Organisation (WHO) standards which causes potential cytotoxicity.

Aim: To evaluate the effect of *Azadirachta indica* leaf extract and the combination effect of *Azadirachta indica* leaf extract (Neem) with Silymarin against SB induced hepatorenal toxicity in adult male albino wistar rats.

Materials and Methods: This experimental interventional animal study was conducted at Central Animal House at Aarupadai Veedu Medical College and Hospital, Puducherry, India from July 2021 to August 2021 for a period of 14 days. Total 30 male wistar rats were randomised into five groups with six rats into each group. The groups were Group 1 Control received only distilled water, Group 2 SB 200 mg/kg bw (SB) alone, Group 3 SB+Silymarin 100 mg/kg bw, Group 4 SB+ *Azadirachta indica* aqueous extract 400 mg/kg bw, Group 5 SB+Silymarin 100 mg/kg bw+*Azadirachta indica* aqueous extract 400 mg/kg bw for 14 days through oral gavage. Doses and duration were determined based on previous studies. Blood was drawn from a retro-orbital puncture, animals were sacrificed by euthanasia, a part of Liver and Kidney samples were sent for histopathological examination-cell structure, integrity, inflammation. Biochemical parameters of liver function tests-Serum Alanine Transaminase (ALT), Serum Aspartate aminotransferase (AST), Serum Alkaline Phosphatase (ALP) and Kidney function tests: urea, creatinine and uric acid were measured in serum. Statistical analysis were done by one-way Analysis of Variance (ANOVA) followed by Dunnet's post-hoc test used for intergroup comparison, p-value <0.05 considered to be significant.

Results: There was a significant increase in the activities of liver enzymes ALT, AST, ALP and kidney function (creatinine, uric acid) in the SB alone treated group when compared with the control group. Hepatorenal protection of Neem extract was shown by significant decrease in liver and renal parameters which was comparable to that of control and Silymarin standard drug. Combination of silymarin and neem showed significant protection in liver (ALT, AST, ALP) and kidney function (urea, creatinine, uric acid) when compared to neem alone treated group. All the results were substantiated by histopathological examination of liver and kidney tissues.

Conclusion: This study suggests effect of *Azadirachta indica* leaf extract with Silymarin possess hepatorenoprotective effect against SB induced damage in rats. Combination effect of *Azadirachta indica* leaf extract with Silymarin significantly proved the hepatorenal protectivity when compared with Neem alone treated group.

Keywords: Animal study, Food preservative, Hepatotoxicity, Neem, Silymarin

INTRODUCTION

The rapid urbanisation of people from rural areas to urban centres and the growing size of middle-class population and the growing attitude to western lifestyle, technological breakthrough are the main factors in the growth of canned foods. India's packaged food market size is estimated to reach 3.4 billion by 2027, growing at Compound Annual Growth Rate (CAGR) of 4.6% during the forecast period of 2022-2027. Various food items like savoury snacks, breakfast cereals, processed meat, frozen sweet corn and readymade meals are readily available in retail stores in their packaged forms. By keeping the food fresh and intact, preservatives have to be added in addition to that it also prevents adulteration practices [1]. SB is a chemical preservative which inhibits the activity of microorganisms in very low concentration and has been generally recognised as safe food preservative [2]. The current maximum usage level permitted is 0.1% in food [3]. SB inhibits the growth of bacteria, yeast, and mould [4]. SB in the mitochondria of liver cells is metabolised by binding to the amino acid glycine and excreted as hippuric acid from the urine. Glycine excretion from the body indicates impaired function of the liver in metabolic process in which glycine is essential. In addition, low glycine levels in the body can reduce creatinine levels, glutamate, urea and uric acid in the urine and increases the levels of these substances in the blood [5]. Though SB is known for its detrimental effects on health, it cannot be completely neglected in this modern era. Hence, present study was done to study the effects of SB on organ dysfunction and how it can be attenuated by various drug therapies.

Several natural products like silymarin, grape fruit, spirulina etc., has been shown to have hepatoprotective effect against various toxicities [6]. Silymarin obtained from Silybum marinum-A milk thistle seed, is one of the widely used drug which has antioxidant, immunomodulatory, antifibrotic, antiproliferative, and antiviral properties that protects the liver from the free radical damage produced by various drug toxicities [7,8]. Therefore, hepatoprotectives are being searched all over the world with greater efficacy and lesser side-effects which focuses mainly on plant based drugs.

Neem (*Azadirachta indica*) leaf extract is well known for its medicinal purposes. Neem leaf extract is the most useful traditional medicine is a source of many therapeutic agents in the Indian culture and grows well in the tropical and semi-tropical countries [9]. In indigenous system of medicine every part of neem tree is used as medicine. Its extracts have antiviral, antibacterial, antifungal, antihelminthic, antidermatic, anti-inflammatory properties and immunomodulatory effects [10]. These activities of neem are due to presence of compounds like nimbidin, sodium nimbidate, geduinin, nembolide, cyclic trisulphide, polysaccharides, polypeptidoglycans [11]. There are several studies showing hepatorenal protectivity of *Azadirachta indica* leaf extract [12,13], but the data is scarce or not adequate enough against its effect on SB induced changes in liver and kidney. The aim of the present study was to investigate the effect of *Azadirachta indica* leaf extract when administered alone and in combination with silymarin against SB induced hepatorenal toxicity in adult male albino wistar rats.

MATERIALS AND METHODS

This interventional experimental animal study was conducted in Central Animal House at Aarupadai Veedu Medical College, Puducherry from July 2021 to August 2021 for a period of 14 days. The study was carried out in accordance with established Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines [14] on the care of laboratory animals following approval by the Institutional Animal Ethical committee. (Certificate attached AVMC/IAEC/2019/07/25/04).

Inclusion criteria: Healthy adult male albino wistar rats having 150-250 grams in weight were included in the study.

Exclusion criteria: Rats with any signs/symptoms of illness and female rats were excluded from this study.

Animals were acclimatised in the experiment room for a period of seven days before dosing. They were housed in polypropylene cages with ad libitum pellet feed and filtered drinking water. The environment was maintained at a temperature of 25±2° Celsius with Relative Humidity around 45-55%. The noise levels were below 80 decibels and light intensity around 160-200 lux and grouping of animals were assigned by simple random sampling method to five groups (six animals in each group) total 30 rats, using the online GraphPad random number generator [15] and the sample size of 30 animals was calculated using G Power version 3.1 software with an effect of 1 level of significance at 5% and 90% power [16]:

Group 1-Control, received distilled water.

Group 2-Sodium Benzoate (SB) 200 mg/kg bw (SB) alone,

Group 3-SB+Silymarin 100 mg/kg bw,

Group 4-SB+*Azadirachta indica* aqueous extract 400 mg/kg bw,

Group 5-SB+Silymarin 100 mg/kg bw+*Azadirachta indica* aqueous extract 400 mg/kg bw. All drugs were administered orally for 14 days through oral gavage.

Study procedure: Urea, Uric acid, Creatinine and enzyme diagnostic kits (ALT, AST, ALP) were obtained from JEEV Diagnostic Pvt., Ltd., Puducherry, India. Standard procedure as specified in the kit literature was followed-Serum ALT (International Federation of Clinical Chemistry method, without Pyridoxal Phosphate activation), Serum AST (IFCC method, without PLP activation), Serum ALP (IFCC method, with PLP activation), Serum Albumin (Bromocresol green method), Serum Protein (Biuret method), Serum Urea (Urease Glutamate Dehydrogenase, Ultraviolet (UV) method), Serum Creatinine (Sarcosine Oxidase method), Serum Uric acid (Uricase-peroxidase method). SB and Silymarin both were obtained from Subra Scientifics, Puducherry, India.

Fresh samples of *Azadirachta indica* (Neem) leaves were collected at farm settlement, at Puducherry, India. The plant was identified at Department of Botany, Department of Biological Science, Pondicherry University. The leaves of *Azadirachta indica* 500 g were air dried to constant weight at room temperature after which they were pulverised. The leaf powder was homogenised in six volumes of 80% methanol for 72 hours after which it was filtered through Whatman No.1 Filter paper [17]. The crude extract was recovered following removal of solvent on a water bath. The concentrated extract was allowed to dry at room temperature after which serial dilution of extract was prepared.

Phytochemical screening: Phytochemical tests were carried out on the aqueous extracts of the sample using standard procedures [18]. Phytochemical screening not only helps to reveal the constituents of plants but also helps in searching for the bioactive agents which can be used for the synthesis of useful drugs [19].

Experimental animals: Male albino wistar rats free of specific pathogens were purchased from Biogen Attibele, Bangalore, India and kept in the Central animal house of Aarupadai Veedu Medical College. Rats were 8-12 weeks of age with the body weight of ranging from 150-250 gm. The rats were kept in ventilated cage at optimum temperature 25±2ºC and 12 hour light/dark cycle and fed with rat pellet and water ad libitum.

All the drugs and chemicals were given orally once a day for all groups throughout the 14 days duration of study based on the body weight of each rat. Hence, it is a subacute toxicity study and the duration and dosage of this experiment is based on the previous study [20]. Average body weight of each group was taken recorded daily and administration of drugs was done using oral gavage.

Preparation of biochemical parameters and histopathology of liver and kidney of rats: After 14 days of administration of drugs in the rats, blood samples and part of liver and kidney tissues were taken on 15th day. Blood samples were collected at retro-orbital sinus of rats and sent for biochemical parameters such as liver and kidney function tests. Rats were then euthanised in CO_2 chamber and a part of liver and kidney were removed from each rat, washed with normal saline and preserved in 10% formalin in separately labelled specimen collection jars and sent for histopathological examination. After one week liver and renal tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 µm section, stained with haematoxylin-eosin dye and then observed under photomicroscope at different magnifications (10x, 40x, 100x). Appreciable changes were noted and subjective grading [20] was done by the pathologist based on a standard classification.

STATISTICAL ANALYSIS

Data were entered and analysed using Statistical Package for the Social Sciences (SPSS) Software (v21.0) by one-way ANOVA and results were expressed as mean±Standard Deviation (SD). Significance of difference between groups was further analysed with Dunnett's test for post-hoc comparisons. The p-value of <0.05 was considered statistically significant.

RESULTS

Body weight: The body weight of the standard drug group (SB+Neem) at the start (198.67±10.89) and end of the experiment (223.8±14.30) shows that there was no significant changes in body weight. This shows that there was no statistically significant (p-value-0.543 and 0.072) difference in body weight of rats at the start and end of the experiment. Hence, it was also observed that that body weight doesn't show any changes in accordance to SB toxicity and treatment groups (*Azadirachta indica* aqueous leaf extract and Silymarin) [Table/Fig-1].

Effect of *Azadirachta indica* on liver enzymes: The present study shows that there was a significant rise in serum liver enzymes in SB treated group, proving that SB has the capability

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	p-value
Initial body weight (gm)	206.83±5.037	211.67±13.75	214.83±21.33	198.67±10.89	208±16.5	0.543
Body weight at the end of experiment (gm)	251.33±15.769	241.33±22.801	213.33±34.892	223.83±14.303	226.17±24.053	0.072

[Table/Fig-1]: Body weight at the start and end of experiment.
One-way ANOVA were used and results were expressed as Mean±SD followed by Dunnett's test for post-hoc comparisons and p-value of <0.05 considered to be statistically significant.

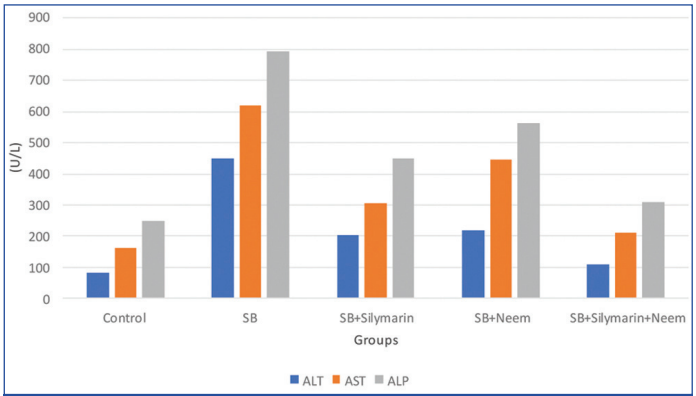
to produce toxicity. While, giving SB with Silymarin and SB with Neem leaf extract, Silymarin treated group has slight significant decrease of ALT, AST, ALP when compared with Neem leaf extract. Comparing the Silymarin alone and Neem leaf extract alone treated group with the combinedly given group shows a significant decrease of liver enzymes (ALT, AST, ALP) proves the combination effect works well. There were no significant changes in serum albumin and total protein (p-values-0.5 for serum albumin and 0.1 for total proteins) in the total experiment. Combination effect of Neem leaf extract with Silymarin gives better results in decreasing the liver enzymes when compared with other treatment groups [Table/Fig-2,3].

and Silymarin has same effect on reducing creatinine and urea levels. Combination effect of Neem leaf extract with Silymarin gives better results when compared with other treatment groups [Table/Fig-4,5].

Histopathological results: Histopathological examination reveals appreciable changes were noted in liver and kidney tissues. Liver showed inflammation, necrosis, severity of hepatitis and regenerative changes. Kidney shows changes in glomeruli and loss of proximal convoluted tubule integrity. It was based on the subjective grading by pathologist as mentioned in the previous study [Table/Fig-6-9] [20].

Groups	Treatment	ALT (mg/dL)	AST (mg/dL)	ALP (mg/dL)	Total protein (mg/dL)	Albumin (mg/dL)
1	Control	82.83±4.2	161.17±9.6	248.67±16.3	5.1000±0.7	1.8833±0.2
2	Sodium Benzoate	448.67±35.0*	619.67±15.9*	792.33±17.3*	4.6833±0.4	1.3167±0.2
3	SB+Silymarin	202.50±10.9#	306.33±9.9#	451.00±23.2#	5.6167±0.5	1.4833±0.4
4	SB+Neem	218.50±19.1$	443.67±26.8$	562.17±21.8$	5.1500±0.2	1.6833±0.4
5	SB+Neem+Silymarin	108.67±7.2¥	210.67±8.2¥	311.17±7.7¥	4.9667±0.4	1.6667±0.4

[Table/Fig-2]: Effect of *Azadirachta indica* leaf extract on liver parameters.
One-way ANOVA were used and results were expressed as Mean±SD followed by Dunnett's test for post-hoc comparisons and p-value of <0.05 considered to be statistically significant.
*p <0.001-when compared with the control group; #p<0.001-when compared with SB alone treated group; $p<0.001-when compared with SB alone treated group; ¥p <0.001-when compared with the neem alone treated group

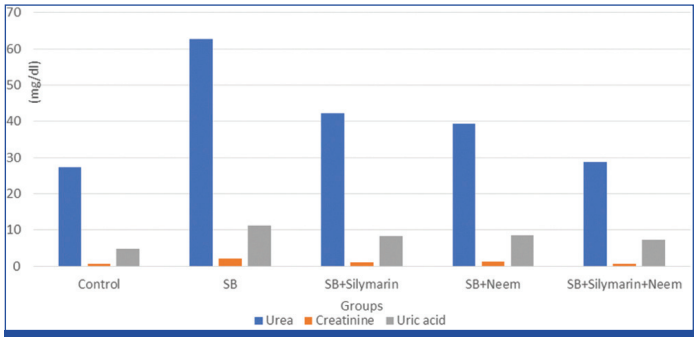


[Table/Fig-3]: Effect of *Azadirachta indica* leaf extract on liver enzymes.

Effect of *Azadirachta indica* on renal functions: Present study showed that SB also have had an effect on renal functions by increasing the serum levels of Urea, Creatinine and Uric acid levels which shows to be SB causes nephrotoxicity. In the treatment groups Neem leaf extract has slight better effect when comparing with Silymarin while comparing the urea alone. But there was some slight difference noted in creatinine and uric acid levels. It shows that Neem leaf extract

Groups	Treatment	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
1	Control	27.33±4.0	0.6667±0.1	4.8667±0.8
2	Sodium Benzoate	62.67±6.8*	2.2167±0.4*	11.2500±1.1*
3	SB+Silymarin	42.17±6.4#	1.1667±0.5#	8.3667±0.4#
4	SB+Neem	39.33±3.5$	1.2500±0.5$	8.6167±0.5$
5	SB+Neem+Silymarin	28.83±0.4¥	0.7167±0.2¥	7.3000±0.3¥

[Table/Fig-4]: Effect of *Azadirachta indica* leaf extract on renal parameters.
One-way ANOVA were used and results were expressed as Mean±SD followed by Dunnett's test for post-hoc comparisons and p-value of <0.05 considered to be statistically significant.
*p<0.001-when compared with the control group; #p<0.001-when compared with SB alone treated group; $p<0.001-when compared with SB alone treated group; ¥p<0.001-when compared with the neem alone treated group



[Table/Fig-5]: Effect of *Azadirachta indica* leaf extract on renal parameters.

Groups	Treatment	Interpretation
1	Control	Section of tissue from the Liver is histologically normal.
2	Sodium Benzoate	Grade 2, 3-hepatitis Loss of tubal cell integrity
3	SB+Silymarin	Grade 1 hepatitis with resolution
4	SB+Neem	Mild hepatitis with resolution
5	SB+Neem+Silymarin	Regenerative changes seen Hyperplasia and hypertrophy of hepatocytes

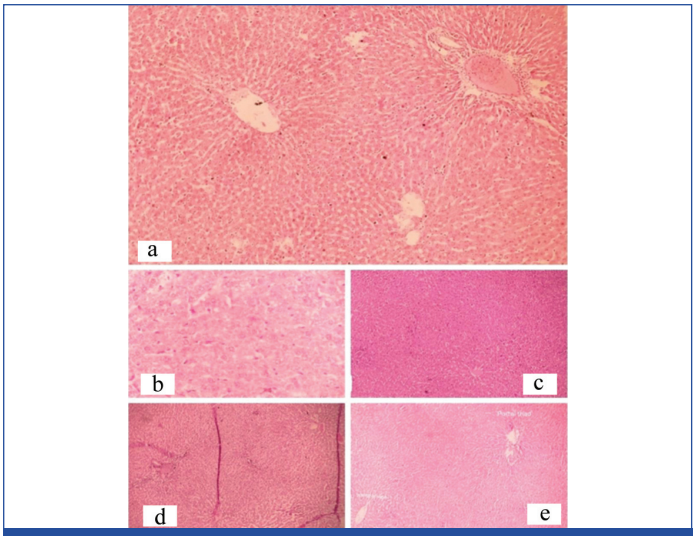
[Table/Fig-6]: Description of histopathological reports-liver.

Groups	Treatment	Interpretation
1	Control	Section of tissue from the kidney is histologically normal
2	Sodium Benzoate	Loss of Proximal convoluted tubular integrity
3	SB+Silymarin	Unremarkable
4	SB+Neem	Unremarkable
5	SB+Neem+Silymarin	Unremarkable

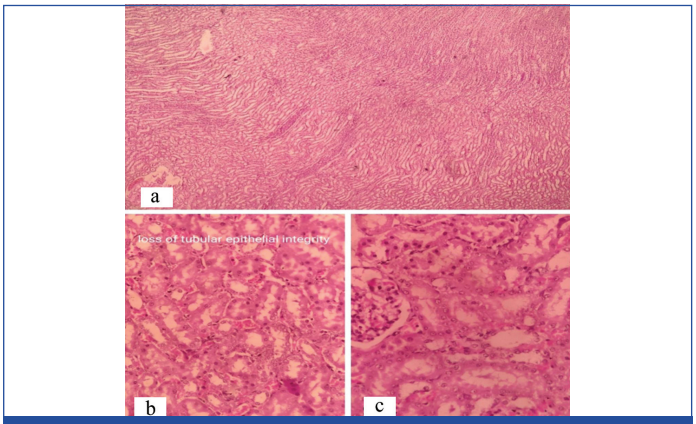
[Table/Fig-7]: Description of histopathological reports-kidney.

DISCUSSION

Liver disorders like jaundice, acute and chronic hepatitis, degenerative disorders and hepatotoxicity are still without



[Table/Fig-8]: Histopathological images- Liver; a) Group 1 Normal Liver morphology showing central vein and portal triad (H&E,10X); b) Group 2 Liver showing architecture loss and necrosis (H&E,40X); c) Group 3 Liver showing hypertrophied and hyperplastic hepatocytes with mild periportal lymphocyte infiltration (H&E,10x); d) Group 4 Liver showing regenerative changes but persistence of sinusoidal dilatation and mild hepatitis (H&E,10x); e) Group 5 Liver showing regenerative changes and no periportal and lobular inflammatory infiltration or necrosis (H&E,10x).



[Table/Fig-9]: Histopathological images- Kidney; a) Group 1 normal cortex, medulla and pyramidal system (H&E,10X); b) Group 2 showing loss of proximal convoluted tubular epithelial integrity (H&E,100X); c) Group 5 showing no change in renal glomeruli, tubules and vessels (H&E,100X).

permanent curable treatment modalities. Many drugs and chemicals can injure the liver [21] and the severity of damage depends on both the dose and duration of exposure of the toxin. Elevated liver enzymes along with jaundice are associated with increased mortality and need for liver transplant [22]. Food preservatives like SB which are consumed regularly causes major liver dysfunction where medicinal plants come in rescue to counter the toxicity due to such insults. Neem is one of those candidate plants and the components of the neem tree, like bark, seed, leaf, fruit, gum, oil contain substances offering some impressive therapeutic applications. However, there has always been a need for more studies that can provide a clear picture for the effect of these herbs against various toxicities. In the present study, the significant change in liver enzymes such as ALT, AST, ALP observed in the rats due to administration of SB shows toxicity has been produced by elevating these enzymes levels. Studies have shown that high level of serum ALT, AST, ALP can result in liver damage [23]. This is because of the SB intoxication results in leakage of cytosolic enzymes into the blood stream due to lipid peroxidation and can damage the cellular membrane [24]. Serum ALT and AST levels determines to be used in large assessment of liver damage. SB causes damage to the hepatocyte (the liver cell) and the crista in the mitochondria can be lost due to the toxicity, leading to cytoplasmic cell injury and cell death [25,26]. In this present study, there was no change in albumin and total protein levels which was measured in all the groups of rats which goes in concordance with a previous study [27]. This clearly shows that toxicity is not enough to alter the protein synthesis in the liver.

Renal enzymes such as urea, creatinine and uric acid are known to have significant raise in their levels showing against when SB is administered. SB alone has the capacity to induce Proximal tubular, glomeruli damages and can cause necrosis, in severe case it can be leading to congestion and calcification [28]. This shows that kidney function has been lost because kidneys are the mainstay in clearance mechanism and can clear the waste products. Urea, uric acid and creatinine are known to be the waste products of metabolism. This had showed that significant raise in blood stream indicates that kidneys had impaired clearance mechanism and there is a defective in the kidney function. This study shows that SB has the tendency to produce toxicity in liver and kidney when administered in the given dose which goes concordance with a previous study [29].

SB has rapid absorption and metabolism as well as quick excretion of metabolites in the body. So, no weight changes are known to be noted. Benzoate conversion to Hippurate occurs within the mitochondrial matrix in two steps. Benzoate enters the mitochondria and is converted to benzoyl CoA (reaction 1) by an ATP-dependent acid: CoA ligase. Benzoyl CoA is subsequently converted to Hippurate (reaction 2) by glycine N-acyltransferase, and then exits the mitochondria. Studies have shown that body weight was altered while giving SB in different dosages [30]. In this study, there was no significant change in body weight was noted which was similar to that of a previous study [31]. It shows that SB has the property of altering body weight only when given in particular dose and duration.

Azadirachta indica (Neem) leaf extract which shows a protective agent against SB induced liver and kidney toxication in the present study which could be due to protective components of the neem like nimbin, nimbidin, azadirichtin, and limonoids. The hepatoprotective activity of neem leaf extract acts by inhibiting the aromatase activity of cytochrome p450 thereby favouring liver regeneration. Neem leaf extract also have antioxidant property and free radical scavenging capacity; thus by stopping the liver damage and favours of liver regeneration [32]. This study shows that SB treated with neem has shows significant reduction in liver and renal enzymes favouring hepatocyte regeneration which is similar to a previous study [33].

This study finding also reveals that silymarin protectivity plays a role in regeneration of hepatocyte favouring hepato protectivity. Silymarin has the capacity of control of free radicals. The free radicals thus causing damages to the cellular membranes and cause lipoperoxidation [34]. The protective effect of silymarin is to be the inhibition of cyclooxygenase cycle, production of free radicals and leukotrienes. Thus, silymarin protects by increasing hepatic protein synthesis. Also, helps in the stimulation of ribosomal RNA polymerase and subsequent protein synthesis, leading to enhanced hepatocyte regeneration [35].

So, combination of *Azadirachta indica* (Neem) leaf extract with Silymarin shows beneficial effects in treating SB induced hepatorenal toxicity in wistar rats when compared to their individual drug administration as described in their individual previous studies [36,37]. Clinically, it signifies that it will improve the patient healthcare as the liver is one of the vital organs, plays a role in detoxification, hence, it will helps in liver related diseases such as liver cirrhosis, hepatitis, non alcoholic fatty liver disease, and kidney related diseases such as glomerulonephritis, urinary tract infections, chronic kidney disease.

Limitation(s)

This study should be done in a longer duration with different dosage levels in other animal species with a larger sample size to know the efficacy of drugs. If proven, this can be given as a safe herbal supplement for all hepatic and renal dysfunction patients.

CONCLUSION(S)

In summary, findings from the present study suggest that *Azadirachta indica* leaf extract at 400 mg/kg bw displays remarkable hepatoprotective effect against SB induced toxicity in experimental rats, possibly because of the presence of bio-active compounds which reduce oxidative stress by scavenging toxic radicals. Findings of the present study also suggest that *Azadirachta indica* leaf extract with silymarin has more beneficial effects against SB induced hepatorenal toxicity. However, more detailed studies are still required to establish the safety, efficacy, and active constituents of this plant to achieve better outcomes in clinical treatments.

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PARTICULARS OF CONTRIBUTORS:

1. Associate Professor, Department of Pharmacology, Aarupadai Veedu Medical College and Hospital, Puducherry, India.
2. MBBS Student, Aarupadai Veedu Medical College and Hospital, Puducherry, India.
3. Associate Professor, Department of Pathology, Aarupadai Veedu Medical College and Hospital, Puducherry, India.
4. Professor and Head, Department of Biochemistry, Aarupadai Veedu Medical College and Hospital, Puducherry, India.
5. Associate Professor, Department of Pharmacology, Aarupadai Veedu Medical College and Hospital, Puducherry, India.
6. Associate Professor, Department of Pharmacology, Aarupadai Veedu Medical College and Hospital, Puducherry, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Deepa Kameswari,
Associate Professor, Department of Pharmacology, Aarupadai Veedu Medical College and Hospital, Kirumambakkam-607402, Puducherry, India.
E-mail: docpdeepa@gmail.com

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