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Research Article

COMPARATIVE STUDY OF THE HEPATOPROTECTIVE ACTIVITIES OF *VITEX NEGUNDO* LINN. AND *MORINGA OLEIFERA* LAM. IN PARACETAMOL INDUCED HEPATOTOXICITY IN EXPERIMENTAL ANIMALS

Mangala Lahkar , **Bhaskarjyoti Thakuria**
Department of Pharmacology, Gauhati Medical College, Bhangagarh, Guwahati-781032
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Corresponding Author's email: bhaskarthakuria4u@gmail.com

Abstract: Aims: To evaluate the hepatoprotective activity of *Vitex negundo* Linn. and *Moringa oleifera* Lam. In paracetamol induced hepatotoxicity in rats. Materials and Methods: Liver damage was induced in 42 albino rats by paracetamol (1 g/kg P.O.) once daily for a period of 7 days. Silymarin was taken as the standard drug in a dose of 25 mg/kg P.O. Albino Wistar rats (150-200 g) were divided into groups containing 6 rats each. The various doses of *Vitex negundo* (100 and 300 mg/kg/day) and *Moringa oleifera* (200 and 800 mg/kg/day) along with Silymarin were adminstered for a period of 14 days. All the animals except the control group received paracetamol for the last 7 days. The animals were sacrificed on the 14th day and blood collected by cardiac puncture. The serum was used for estimation of liver enzymes, protein and bilirubin. These were supplemented by pentobarbitone induced sleeping time and liver histopathology. Statistical analysis used: Data was analyzed by one-way ANOVA followed by Dunnet's T test.Results: Both *Vitex negundo* and *Moringa oleifera* Lam. resulted in statistically significant ($p<0.05$) reduction in liver enzymes and proteins in a dose dependent manner but *Moringa oleifera* showed greater activity. Pentobarbitone sleeping time and liver histopathology yielded consistent results. Conclusion: Ethanolic extracts of *Vitex negundo* and *Moringa oleifera* shows hepatoprotection but *Moringa oleifera* shows more activity.

Key words: Hepatoprotection; Paracetamol; *Vitex negundo*; *Moringa oleifera*; Silymarin

INTRODUCTION

Hepatic injury can be caused by ingestion, inhalation or parenteral administration of pharmacological and chemical substances. The damage to the hepatocytes has been implicated to the binding of N-acetyl benzoquinoneimine to hepatocytes leading to enzymatic dysfunction and structural and metabolic disarray.The reduction of glutathione renders hepatocytes susceptible to oxidative stress and apoptosis[1].The hepatoprotective effect of various drugs has been studied on various paracetamol induced hepatotoxicity models. Likewise, various plant extracts have been studied in paracetamol induced hepatotoxicity models.

Vitex negundo Linn. (verbenaceae), a large aromatic shrub with typical five foliate leaf pattern has been claimed to possess many medicinal properties[2]. It is commonly known as the 'five-leaved chaste tree' or 'Monk's Pepper'. The leaves contain an alkaloid nishindine, flavonoids like flavones, luteolin-7-glucoside, casticin and other constituents like vitamin C, carotene, benzoic acid, β-sitosterol and C-glycoside. Leaves are aromatic, bitter, acrid, astringent, anodyne, antinflammatory, antipyretic or febrifuge, tranquilizers, bronchial smooth muscle relaxants, antiartritic, antihelminthic and vermifuge[3].The plant has been extensively studied for its anti-inflammatory and analgesic activity in the past[4,5]. Researchers have reported that qualitative phytochemical investigations have shown positive for flavonoids, glycosides, tannins, terpenoids and it has also been postulated that the flavonoid constituent of the plant possesses antioxidant properties and is useful in the treatment of liver damage.

*Moringa oleifera Lam.*is the most widely cultivated species of the genus Moringa, the only genus in the family Moringacea. The leaves of the plant act as a good source of natural antioxidants due to presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolic and carotenoids. The immature green pods called "drumstick" are probably the most valued and widely used part of the tree. They are commonly consumed in India. The seeds are sometimes removed from more mature pods and eaten like peas or roasted like nuts. The flowers are edible when cooked. The roots are shredded and used as a condiment in the same way as horseradish. The leaves are highly nutritious, being a significant source of beta-carotene, Vitamin C, protein, iron,

and potassium[6]. Almost all parts of this plant have been used for the treatment of various ailments in indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders[7]. Based on the uses and phytoconstituents of both these plants they were selected for study of their hepatoprotective study.

MATERIALS AND METHODS

The present study has been carried out in the Department of Pharmacology, Gauhati Medical College and Hospital, Guwahati to study the hepatoprotective activity of *Vitex negundo* Linn. And *Moringa oleifera* Lam. in paracetamol induced hepatic damage in albino rats.

The leaves of *Vitex negundo* Linn. were procured from a rural locality near Guwahati & were used in the study. The leaves of *Moringa oleifera* Lam. were collected from local market in Guwahati and were used in the study.Voucher specimens of both plants were preserved for future identification. Both the plant leaves were air dried & subjected to the following procedures. The air dried leaves were finely powdered in an electrical grinder. 500 grams of powdered leaves was then extracted using 1000 ml of 80% hydroethanolic solvent containing Ethanol & water in the ratio 80:20 (80% ethanol & 20% water) in a Soxhlet extractor & was allowed to remain for 15 minutes in a tightly covered container. The liquid extract obtained was then collected in a flask and filtered. The extract was flask evaporated by using controlled temperature (hot water bath temperature 40-50˚C) until the solvent part was evaporated[8] . The extract obtained was then collected in glass petri dishes, further dried in a vacuum dessicator and was finally stored in air tight glass containers in a refrigerator at 2-8˚C for use in the experiments. A final yield of 90.5 grams i.e. 18.1 % w/w with respect to the original air dried powder was obtained in *Vitex negundo*. In case of *Moringa oleifera* a final yield of 82.6 grams i.e., 16.52% w/w with respect to the original air dried powder was obtained.

The study was conducted after obtaining approval from Institutional Animal Ethical Committee (No.MC/32/2012/7) and animals used in this study were treated and cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) (Regd. No.: 12/GMCH/CPCSEA/351).

Acute Toxicity Study:

The doses of the drugs were chosen by arbitrarily choosing two different doses from previous studies. We arbitrarily selected doses of the extract above and below that dose and then carried out the preliminary toxicity studies by increasing the doses of the drug. We found that the NOAEL (No Observed Adverse Effect Limit) of the **hydroethanolic extract of *V.negundo*** (HEVN) was found to be 2000 mg/kg /day and the NOAEL of the **hydroethanolic extract of *M.oleifera*** (HEMO) leaves was also found to be 2000 mg/kg/day carried out as per OECD Guideline 425[9].

Drugs used in the study:

Paracetamol was procured from Neon Labs Limited, Thane, Maharashtra. Silymarin was procured from Micro Labs limited, Bangalore, India. Thiopentone was procured from the local market. Enzymes like alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total protein and total bilirubin were assayed using standard kits from 'Synergy Laboratories', India and 'Crest Biosystems', India.

***Study Groups*:**

A total of 42 animals were included in the study with each group consisting of 6 albino rats each. The animals were divided into the following groups.

Group I: Control group (normal saline fed for 14 days)

Group II: Paracetamol induced hepatotoxicity group (Paracetamol p.o. for last seven days)

Group III: Paracetamol induced hepatotoxicity +HEVN (Paracetamol p.o. for the last seven days)

Group III (subgroup A): Paracetamol induced hepatotoxicity + HEVN (100 mg/kg/day p.o. for fourteen days)

Group III (subgroup B): Paracetamol induced hepatotoxicity + HEVN (300 mg/kg/day p.o. for fourteen days)

Group IV: Paracetamol induced hepatotoxicity +HEMO (Paracetamol p.o. for the last 7 days)

Group IV (subgroup A): Paracetamol induced hepatotoxicity + HEMO (200 mg/kg/day p.o. for fourteen days)

Group IV (subgroup B): Paracetamol induced hepatotoxicity + HEMO (800 mg/kg/day p.o. for fourteen days)

Group V: Paracetamol induced hepatotoxicity + silymarin (25 mg/kg/day PO)[10] (Paracetamol p.o. for the last seven days)

Group III, **Group IV** and **Group V** received HEVN, HEMO, and silymarin respectively for 14 days and each group of chronic study received paracetamol (1 g/kg p.o.) for the last seven days.

The animals were adminstered paracetamol p.o. (1 g/kg/day)[11] in the study for the last seven days for induction of subacute hepatotoxicity.In the study groups on the 13th day thiopentone sodium (37 mg/kg i.p.) was adminstered to the animals of each group

and the sleeping time was noted.. Thiopentone induced sleeping time was calculated from the interval between loss and gain of righting reflex.In the study groups, on the 14th day, i.e., after adminstration of the last dose of paracetamol p.o., the animals were sacrificed under light ether anaesthesia by cervical dislocation and blood samples were collected by cardiac puncture.The liver was dissected out and was carefully examined, regarding the size and shape, colour and presence or absence of any nodule. The weight of each liver was taken and fixed in 10% formalin solution and sent for histopathological examination[11]. The blood samples collected by intracardiac puncture were kept for 30 minutes without disturbing. The clot was dispersed with a glass rod and centrifuged for 15-20 minutes at 1500 rpm to separate serum.The serum of each animals in every group was estimated for total protein, total bilirubin, SGOT, SGPT, ALP. The sacrificed animals were put in yellow bag and sent for incineration. Biochemical estimation was done using a computer-assisted spectrophotometer "Thermo scientific spectrophotometer".

RESULTS

Results obtained from the study are summarized in the following tables. Results of estimations were expressed as Mean ± Standard Error of Mean (S.E.M.). The statistical significance was analyzed using One Way ANOVA followed by Dunnet test for multiple comparison.

TABLE 1: ALT and AST levels in the different study groups at the end of the experiment

Groups	AST (U/L)	ALT(U/L)
Control	39.76±1.00	38.19±2.02
Paracetamol	153.15±2.13*	158.29±3.52*
HEVN 100	111.04±1.27**	112.58±1.60**
HEVN 300	103.45±0.88**	108.65±0.89**
HEMO 200	106.23±0.95**	112.74±0.66**
HEMO 800	101.31±1.12**	109.07±1.05**
Silymarin	76.73±3.27**	80.03±1.90**

TABLE 2: ALP and Bilirubin levels at the end of the experiment

	ALP (U/L)	Bilirubin(mg/dl)
Control	131.10±1.82	0.49±0.06
Paracetamol	416.81±7.91*	16.62±0.71*
HEVN100	304.62±1.67**	9.13±0.37**
HEVN300	287.97±4.10**	5.50±0.27**
HEMO200	319.92±3.57**	8.57±0.31**
HEMO800	282.83±2.85**	5.35±0.24**
Silymarin	206.46±5.78**	4.12±0.21**

(Results expressed in Mean±S.E.M.; n=6; one-way ANOVA followed by Dunnet's test, * P<0.05 when compared with control group., ** P<0.01 when compared with paracetamol intoxicated group)

Tables 1-2 demonstrates the variations in serum enzyme level following drug treatment for 14 days. On day 14, all the biochemical estimations such as ALT, AST, ALP were found to be significantly increased in all groups except control and there was a significant decrease in the values in the extract-treated and the silymarin groups. From Table 2 bilirubin levels show a significant decline in values in the extract-treated and the silymarin groups when compared with the paracetamol group. From Table 3 a decline in total protein was seen in the paracetamol group with a significant ($p<0.05$) difference with the treated groups.

TABLE 3: Values of total protein, absolute liver weight and sleeping time induced by phenobarbitone at the end of the experiment.

Groups	Total Protein (gm/dl)	Absolute Liver Weight (gm/100gm body wt).	Sleeping Time (in minutes)
Control	6.07±0.33	6.48±0.06	19.93±0.44
Paracetamol	2.37±0.22*	9.21±0.15*	253.53±6.34*
HEVN100	4.30±0.17**	7.08±0.08**	124.15±2.13**
HEVN300	4.76±0.27**	6.94±0.17**	115.99±0.60**
HEMO200	4.31±0.17**	7.17±0.17**	89.72±0.99**
HEMO800	4.86±0.26**	6.96±0.15**	99.29±1.04**
Silymarin	5.16±0.50**	6.56±0.10**	48.03±1.95**

(Results expressed in Mean±S.E.M.; n=6; one-way ANOVA followed by Dunnet's test, * P<0.05 when compared with control group., ** P<0.01 when compared with paracetamol intoxicated group)

From Table 3 an increase in liver weight was seen but the treated groups demonstrated significantly lower liver weights when compared with paracetamol group. Sleeping time was prolonged in the paracetamol group. The treated groups showed no significant ($p<0.05$) increase in the duration of sleep induced by thiopentone when compared with the paracetamol group.

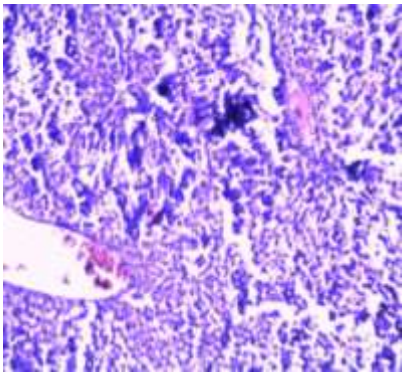
DISCUSSION

Paracetamol induced hepatotoxicity is due to the depletion of glutathione which fails to oxidize the reactive metabolites N-Acetyl p-Benzoquinoneimine (NAPQI). Besides NAPQI also reacts with protein leading to oxidative stress and protein dysfunction. The resulting mitochondrial damage leads to necrosis and apoptosis of hepatic cells[12]. Later the intracellular constituents released due to cellular necrosis are recognized by the activated innate immune system leading to activation of inflammatory protein complex activating proinflammatory cytokines IL-1β and IL-18[13].

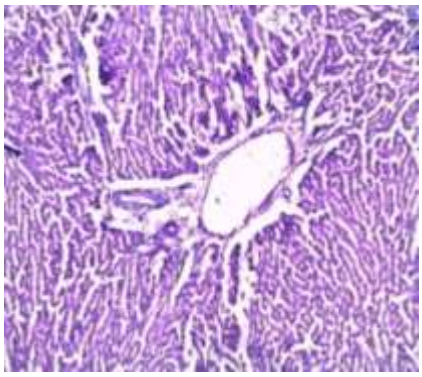
Total serum protein and bilirubin levels reflect the function of the hepatocytes. Decreased levels of total protein indicated failure of the biosynthetic function of the hepatocyte, while increased levels of bilirubin is indicative of defective hepatocellular uptake, conjugation and excretion of bilirubin due to failure of hepatic cell function[14,15] .The results demonstrated that *Vitex negundo* (HEVN) and *Moringa oleifera* (HEMO) caused a significant increase in levels of total protein when compared with Paracetamol treated group while there was a significant decrease in bilirubin levels when compared with the Paracetamol treated group. In this respect HEMO (at dose of 800 mg/kg) resulted in an increased level of total protein at 4.86 gm/dl which was comparable to that of the standard drug Silymarin while that due to HEVN (at dose of 100 mg/kg) showed the least increase at 4.30 gm/dl. In a similar manner HEMO (at dose of 800 mg/kg) showed the maximum decrease in levels of serum bilirubin at 5.35 mg/dl.

Increase in the levels of serum transaminases (ALT and AST) are indicative of cellular leakage and loss of functional integrity of the hepatocytes in the liver. ALP levels are inceased as a result of leakage from bile canaliculi into hepatic sinusoids and is a result of leaky tight junctions and the other hypothesis is that the damaged hepatocytes fail to excrete alkaline phosphatase made in the bone, intestine and the liver[14].HEMO at dose of 800 mg/kg showed the maximum decrease (101.31 U/L) in AST levels and that of ALP (282.83 U/L) when compared to the paracetamol treated group.

Thiopentone induced sleeping time is indicative of liver metabolism since barbiturates are exclusively metabolized in the liver. Due to a preexisting paracetamol induced liver damage the sleeping time will be prolonged as the amount of hypnotic metabolism per unit time will be less. The ability of HEVN and HEMO to reduce the prolongation of thiopentone induced sleeping time in paracetamol induced liver injury is supportive of their hepatoprotective action and this is seen best with HEMO at a dose of 200 mg/kg (89.7 minutes).



(A)



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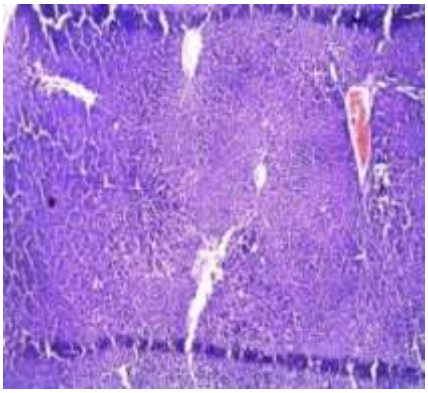

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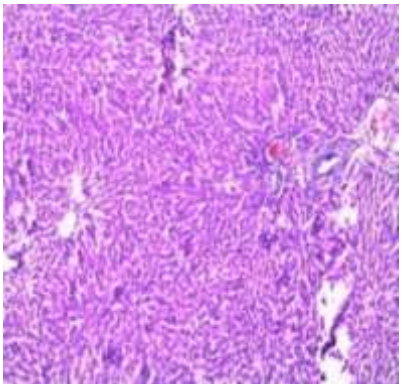

(D)

FIGURES: (A) **Liver Injury** due to paracetamol after 14 days of drug administration showing areas of cellular degeneration (marked with arrow) with dilated & congested vein, (B) Liver section from rats from **Group V** (Silymarin treated standard group/**acute** study) showing intact portal triad, normal hepatocytes and normal blood vessels,(C) Liver section from HEVN treated **Group IIIB** animals showing near normal lobular architecture with congested and dilated sinusoids,(D) Liver section from HEMO treated **Group IVB** animal showing near normal lobular architecture with areas of congestion (arrows) without any signs of necrosis.

Liver weight was increased in all the paracetamol treated groups compared to control. But all the test groups showed a significant reduction in liver weight when compared with paracetamol treated group. The histopathological examination revealed that the paracetamol treated group revealed perivenular inflammatory infiltrate, congested and dilated blood vessels and also areas of centrilobular necrosis with mild inflammatory infiltrate. In rats treated with HEVN and HEMO extracts the groups show no or minimal inflammatory infiltrate although they revealed the presence of congested and dilated central veins and sinusoids. Thus the histopathological study shows a reduction in the degree of necrosis in rats treated with both extracts.

Previously Tasduq et al.[16] studied the activity of flavonoid natural products from the leaves of *Vitex negundo* Linn in a model of carbon tetrachloride (CCl_4) mediated hepatotoxicity and implicated that 2'-p-hydroxybenzoylmussaenosidic acid [negundoside (NG)] (a purified irridoid glycoside from leaves of *Vitex negundo*), is the key flavonoid involved in hepatoprotection. The mechanism of protection involves decreased production of ROS and lipid peroxidation when the CYP2E1 mediated oxidative stress was produced in experimental cells with pro-oxidant as CCl_4. The main mechanism involved in the cytoprotection of NG seems to be its ability to protect the mitochondria against depletion in its membrane potential, an event that is very critical in the loss of cell viability as a consequence of oxidative stress. The same flavonoid may be implicated in protection against paracetamol induced hepatotoxicity.

In previous studies [17,18] parts of the Moringa plant (viz. the roots and the flowers) were found to have hepatoprotective activity and this was proposed by Gilani et al.[18] to be due to quercetin, a well known flavonoid with hepatoprotective activity. In this study where we have taken for evaluation the leaves of the *Moringa oleifera* plant a similar mechanism involving such flavonoids natural products may be implicated in providing a certain degree of hepatoprotection to the rat liver.

CONCLUSION

Based on the results of the present study we can conclude that the hydroethanolic extracts of *Vitex negundo* and *Moringa oleifera* suppresses paracetamol-induced hepatocellular damage. The extract of *Moringa oleifera* showed more activity in this respect. Further investigations with isolated active principles are required to throw more light on this activity.

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