

**IN VITRO AND IN VIVO HEPATOPROTECTIVE EFFECT OF
VITEX NEGUNDO LEAVES**

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Summary

To investigate the methanol extract of leaves of *Vitex negundo* (Verbeneaceae) for its hepatoprotective activity against CCl₄ induced toxicity in freshly isolated rat hepatocytes, HepG2 cells and animal models. Mature leaves of *Vitex negundo* were collected, authenticated and subjected to methanolic extraction. Freshly isolated rat hepatocytes were exposed to CCl₄ (1%) along with/without various concentrations of the methanolic extract of *Vitex negundo* (50–250µg/ml) and the levels of selected liver enzymes were measured. Hepatoprotective activity of the methanolic extract of *Vitex negundo* (50–250µg/ml) based on the protection of human liver derived HepG2 cells against CCl₄ induced damage was determined by MTT assay. Twenty four Wistar strain albino rats (180–200 g) of either sex were used for the *in vivo* investigations. Liver damage was induced by administration of 30 percent CCl₄ suspended in olive oil (1 ml/kg body weight, i.p). The animals were divided into four groups. Group I received the vehicle (Sodium CMC 0.3%). The second group received CCl₄ (1 ml/kg body weight, i.p). Group III received methanolic extract of *Vitex negundo* (200mg/kg body weight), and group IV received the standard drug Silymarin (250mg/kg body weight). The animals received these treatments by the oral route for a period of 7 days. On the seventh day except group I, all other groups received 30 percent CCl₄ suspended in olive oil (1 ml/kg body weight) intra-peritoneal. After 24 h of intoxication, on the 8th day, blood was

collected; serum separated and various biochemical parameters were estimated. The antihepatotoxic effect of the methanolic extract was observed in freshly isolated rat hepatocytes at very low concentrations (50–250µg/ml) and was found to be superior to that of the standard used. A dose dependent increase in the percentage viability was observed when CCl₄ exposed HepG2 cells was treated with different concentrations of the methanolic extract of *Vitex negundo*. The highest percentage viability of HepG2 was observed at a concentration of 250µg/ml. Its *in vivo* hepatoprotective effect at 200 mg/kg body weight was comparable with that of the standard at 250mg/kg body weight. The methanolic extract was able to normalise the biochemical levels which were altered due to CCl₄ intoxication in freshly isolated rat hepatocytes and also in animal models.

Keywords: *Vitex negundo*, hepatoprotective, hepatocytes, HepG2.

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Introduction

Vitex negundo (Verbeneaceae) is a large aromatic shrub up to 4-5 meters in height is found throughout the greater part of India.^[1] In homeopathy, it is being used for the treatment of snake bite^[2]. It is used for its anti inflammatory and analgesic activity^[3]. The plant is also known to possess liver protective effects^[4] and larvicidal activity^[5]. However, the hepatoprotective effect of methanolic extract of the plant *Vitex negundo* has not been investigated and compared between primary hepatocytes, established cell lines and animal models. Hence this present study was intended to investigate the *in vitro* and *in vivo* hepatoprotective effect of the methanolic extract of leaves of *Vitex negundo*.

Materials and Methods

Materials

All chemicals were obtained from SD Fine Chemicals, Mumbai. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), collagenase, insulin, dexamethasone, minimum essential medium (MEM), Ham's F12 medium, Silymarin (Standard) and antibiotics were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ecoline diagnostic kits were purchased from E – Merck, India. The human liver derived HepG2 cell line was obtained from National Centre for Cell Science, Pune, India.

Plant material

Mature leaves of *Vitex negundo* were collected from the fields in and around mavelikara village, Kerala in the month of May 2006. The plant was authenticated by Botanical Survey of India, Coimbatore, Tamil Nadu (Authentication no. BSI/SC/5/17/06-07/Tech.699).

Preparation of the plant extract

The fresh mature leaves (350 g) were subjected to a single extraction in a Soxhlet extractor using methanol (1 L) for 18–20 h. The extract was then concentrated to dryness under reduced pressure and controlled temperature to yield a dark green semisolid mass (24.2 g, 6.91%), which was preserved in refrigerated conditions.^[6]

Preparation of suspensions

The methanolic extract of *Vitex negundo* was dissolved in DMSO and the volume was made up to 10ml with Ham's F-12/MEM to obtain a stock solution of 1mg/ml concentration and stored at -20 °C prior to use. Further dilutions were made to obtain different concentrations ranging from 50–250µg/ml with respective media and used for *in vitro* investigations. A suspension of the standard powder was also prepared (250µg/ml) in a similar manner. The methanolic extract and the standard powder were suspended in sodium CMC (0.3%) in distilled water separately and used for *in vivo* investigations.

Hepatoprotective effect of the plant extract in freshly isolated rat hepatocytes:***Isolation and culture of hepatocytes***

Liver cells were isolated by a modified procedure of Seglen (1979) [7]. The calcium-free HEPES buffer and collagenase solutions were warmed in a water bath (37 °C). The abdomen of the rat was opened under phenobarbital sodium (35mg/kg body weight) anesthesia. A midline incision was made and a loosely tied ligature was placed around the portal vein approximately 5mm from the liver and the cannula was inserted up to the liver and then the ligature was tightened and heparin was injected into the femoral vein (1000 IU). The inferior venacava was cut below the renal vein. Perfusion was performed for 20 min (37 °C) with calcium free HEPES buffer, which contained 1% bovine serum albumin fraction V at a flow rate of 30 ml/min. The liver swells during this time, slowly changing its color from dark red to grayish white. The swollen liver was then perfused with TPVG solution (50 ml) followed by perfusion with calcium free HEPES buffer, which contained additional collagenase solution (0.075%) and calcium chloride (4mM) at a flow rate of 15 ml/min for 20 min. After the perfusion, the lobes were removed and transferred into a sterile petri dish containing calcium-free HEPES buffer and dispersed gently. It was transferred into a sterile conical flask and the crude cell suspension was stirred with the help of a magnetic stirrer for 5 min to release hepatocytes into the solution. The cell suspension was filtered through a nylon mesh (250 μ) and the preparation was centrifuged at 1000 rpm for 15 min. The supernatant was aspirated off and the loosely packed pellet of cells was gently re-suspended in calcium free HEPES buffer. This washing procedure was repeated three times. Cell viability was determined by the Trypan blue dye exclusion method [8]. These isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, 10⁻⁶M dexamethasone and 10⁻⁸ bovine insulin. The cell suspension was incubated at 37 °C for 30 min in a humidified incubator under 5% CO₂.

Carbon tetrachloride induced in vitro hepatocytes injury

Carbon tetrachloride induced hepatocytes injury assay was carried out. After an incubation of 24 h, the hepatocytes were exposed to the fresh medium containing CCl₄ (1%) along with/without various concentrations of methanolic extract of *Vitex negundo* or the medium alone (as normal).

After 60 min of CCl₄ challenge, concentrations of aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphatase (ALP), triglycerides (TGL), total proteins, albumin, total bilirubin and LDH in the medium were measured as an indication of hepatocytes necrosis using Ecoline diagnostic kits ^[9].

Hepatoprotective effect in HepG2 cell line

The screening of hepatoprotective activity was based on the protection of human liver derived HepG2 cells against CCl₄ induced damage ^[10] determined by estimating mitochondrial synthesis using tetrazolium assay ^[11]. HepG2 cells were routinely grown and subcultured as monolayers in DMEM supplemented with 10% newborn calf serum. The experiments in this investigation were conducted with cells that had been initially batch cultured for 10 days. At this stage, the cells were harvested and plated at approximately 30,000 cells/well in 96 well microtitre plates (Nunclon) and left to rest for 24 h at 37 °C in a humidified atmosphere of 5% CO₂. The cells were then exposed to toxicant (medium containing 1% CCl₄) along with/without various concentrations of the methanolic extract of *Vitex negundo* or the medium alone (as normal) ^[10]. At the end of the period, cytotoxicity was assessed by estimating the viability of HepG2 cells by MTT reduction assay ^[11]. After 1 h incubation, the test solution from each well was removed by aspiration and replaced with 50µl of MTT prepared in MEM without phenol red (MEM-PR). The plates were gently shaken and incubated for 3 h at 37 °C in a humidified 5% CO₂ atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 540nm.

In vivo hepatoprotective effect

Colony bred Wistar strain adult albino rats (180–200 g) of either sex were used for the investigations. All the animals were maintained under standard husbandry conditions with food and water *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), KMC, Manipal (No.IAEC/KMC/06/2006-2007). The animals were divided into four groups of six animals in each group. Liver damage was induced by administration of 30 percent CCl₄ suspended in olive oil (1 ml/kg body weight, i.p). Group I received the vehicle (Sodium CMC 0.3%) and served as control and was not treated with the toxicant. The second group served as CCl₄ treated control. Group III received a

suspension of the methanolic extract of leaves of *Vitex negundo* (200 mg/kg body weight), and group IV received the standard (250mg/kg body weight). The animals received these treatments by the oral route for a period of 7 days. On the seventh day except group I, all other groups received 30 percent CCl₄ suspended in olive oil (1 ml/kg body weight) intra-peritoneal. After 24 h of intoxication, on the 8th day, blood was collected in sterile centrifuge tubes and allowed to clot. Serum was separated and used for the estimation of ASAT, ALAT, ALP, TGL, total proteins, albumin, total bilirubin and LDH using Ecoline diagnostic kits^[9, 12].

Histopathological examination

Liver was removed, fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under light microscope. In brief, 4- μ m thick sections of paraffin-embedded mice liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 min. Subsequently, the sections were stained with hematoxylin for 5 min at room temperature. After 15 min, the sections were counterstained with eosin for 2 min, dehydrated in graded alcohol series, washed with xylene, and blocked by rosin. H&E- stained slides were observed under microscope at $\times 40$ magnifications.

Statistical analysis

The statistical analysis was carried out by one way analysis of variance (ANOVA). The values are represented as mean \pm S.E.M. Comparison of mean values of different groups treated with different dose levels of total alkaloid fraction and positive control with normal were estimated by Turkey's Multiple Comparison Test. $P < 0.05$ was considered significant.

Results

Hepatoprotective effects in freshly isolated rat hepatocytes

The effects of the methanolic extract of *Vitex negundo* on freshly isolated rat hepatocytes intoxicated with CCl₄ are recorded in Table 1. A significant increase in the levels of ASAT, ALAT, ALP, total bilirubin, LDH ($P < 0.001$) and a significant reduction in the levels of TGL, total proteins and albumin ($P < 0.001$) were observed in hepatocytes exposed to CCl₄ when compared to normal rats.

Table 1 Effects of treatment of methanolic extract of *Vitex negundo* leaves on the biochemical parameters of CCl₄ intoxicated freshly isolated rat hepatocytes.

Treatment	Concentration	ASAT U/L	ALAT U/L	ALP U/L	Albumin g/L	Total Bilirubin mg/dL	Total Protein g/dL	TGL mg/dL	LDH U/L
Normal	-	15.00 ± 0.46	11 ± 0.39	34 ± 0.01	2.62 ± 0.06	0.20 ± 0.002	1.2 ± 0.04	162 ± 9.08	118 ± 0.005
CCl ₄	1%	84 ± 2.86 ^a	58 ± 0.48 ^a	98 ± 0.53 ^a	0.9 ± 0.01 ^a	0.68 ± 0.03 ^a	0.4 ± 0.06 ^a	71 ± 3.06 ^a	298 ± 0.01 ^a
CCl ₄ (1%) + standard	250 µg	18 ± 0.97 ^b	14 ± 0.83 ^b	36 ± 1.24 ^b	2.14 ± 0.03 ^b	0.28 ± 0.002 ^b	1.14 ± 0.024 ^b	156 ± 12.47 ^b	128 ± 0.02 ^b
	250 µg	25 ± 0.98 ^b	13 ± 0.92 ^{b,d}	34 ± 1.22 ^{b,e}	2.15 ± 0.04 ^b	0.25 ± 0.002 ^{b,d}	1.14 ± 0.02 ^b	159 ± 10.08 ^b	120 ± 0.04 ^{b,c}
CCl ₄ (1%) + methanolic extract	200µg	26 ± 1.39 ^b	14 ± 0.73 ^{b,c}	35 ± 0.66 ^b	2.14 ± 0.07 ^b	0.26 ± 0.002 ^b	1.10 ± 0.03 ^b	156 ± 8.90 ^b	123 ± 0.01 ^b
	150µg	29 ± 1.34 ^b	18 ± 0.51 ^b	37 ± 1.02 ^b	2.09 ± 0.05 ^b	0.29 ± 0.001 ^b	1.09 ± 0.03 ^b	153 ± 9.81 ^b	128 ± 0.02 ^b
	100µg	30 ± 1.76 ^b	20 ± 0.51 ^b	39 ± 0.99 ^b	1.99 ± 0.05 ^b	0.29 ± 0.003 ^b	1.07 ± 0.02 ^b	150 ± 5.52 ^b	130 ± 0.02 ^b
	50µg	32 ± 1.80 ^b	22 ± 0.58 ^b	40 ± 1.63 ^b	1.96 ± 0.04 ^b	0.32 ± 0.003 ^b	1.04 ± 0.03 ^b	147 ± 6.73 ^b	137 ± 0.02 ^b

Number of independent experiments = 3, 5 replicates, mean ± SEM a = P < 0.001, when compared to normal group, b = P < 0.001, when compared to CCl₄ group, c = P < 0.05, d = P < 0.01, e = P < 0.001, when compared to standard.

These cells, when treated along with the methanolic extract of *Vitex negundo*, showed a significant restoration of the altered biochemical parameters towards the normal ($P < 0.001$, when compared to CCl_4 treated group) and is dose dependent. A similar result was obtained when CCl_4 intoxicated hepatocytes were treated with the standard. However, the hepatoprotective effect of methanolic extract of *Vitex negundo* was observed at very low concentrations (50–250 $\mu\text{g/ml}$) when compared to the standard. The decrease in the levels of ASAT, ALAT, total bilirubin and LDH in freshly isolated hepatocytes treated with methanolic extract of *Vitex negundo* at 250 $\mu\text{g/ml}$ was significant ($P < 0.05 - 0.001$, when compared to standard) and more than that produced by the standard at the same concentration.

Table 2 Hepatoprotective activity of the methanolic extract of *Vitex negundo* leaves on CCl_4 intoxicated HepG2 cells.

Treatment	Concentration ($\mu\text{g/ml}$)	% Viability
Control	-	100
CCl_4	-	17.65 ± 2.16^a
CCl_4 + Standard	250	91.54 ± 3.12^b
	250	$93.42 \pm 4.21^{b,c}$
CCl_4 + Methanolic extract	200	$91.15 \pm 3.94^{b,c}$
	150	87.32 ± 3.65^b
	100	82.43 ± 4.01^b
	50	80.05 ± 3.03^b

Average of 5 determinations, 4 replicates

a = $P < 0.001$, when compared to normal cells.

b = $P < 0.01$, when compared to CCl_4 intoxicated cells.

c = $P < 0.01$, when compared to standard treated cells.

Hepatoprotective effects in the HepG2 cell line

The CCl₄ exposed HepG2 cells showed a percentage viability of 17%. These exposed cells, when treated with different concentrations of the total alkaloid fraction of *Vitex negundo*, showed a dose-dependent increase in percentage viability and the results were highly significant ($P < 0.001$, when compared to CCl₄ intoxicated group). The percentage viability ranged between 80–93% at 50–250µg/ml concentration of methanolic extract (Table 2). The increase in percentage viability of the HepG2 cells treated with methanolic extract at 250µg/ml was significant ($P < 0.01$, when compared to standard) and more potent than that produced by the standard at the same concentration.

In vivo hepatoprotective effects

The effects of methanolic extract of *Vitex negundo* on CCl₄ intoxicated rats are recorded in Table 3. Intoxication of rats treated with CCl₄ significantly altered the biochemical parameters when compared with normal control rats ($P < 0.001$). Treatment of methanolic extract of *Vitex negundo* at 200 mg/kg body weight showed a significant decrease in ASAT, ALAT, ALP, total bilirubin, LDH ($P < 0.001$) and a significant elevation in the TGL, total proteins and albumin levels ($P < 0.001$) in serum when compared with CCl₄ treated rats. Standard at 250mg/kg body weight also exhibited similar results. All biochemical findings were positively supported by the histopathological results (Figure 1 - 4).

Histopathology

Figure 01 Normal Liver (vehicle control: Sodium CMC 0.3%)

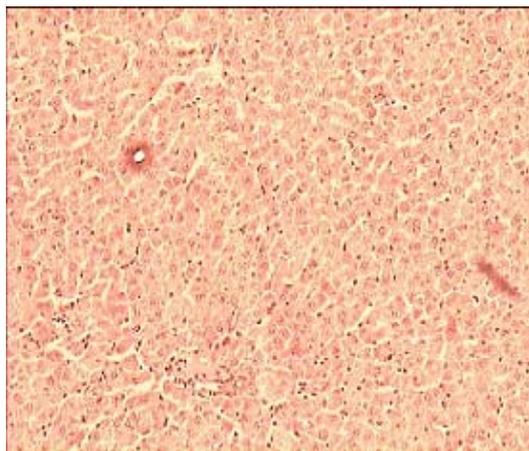


Figure 02 Toxicant treated liver (CCl₄ 1ml/kg body wt)

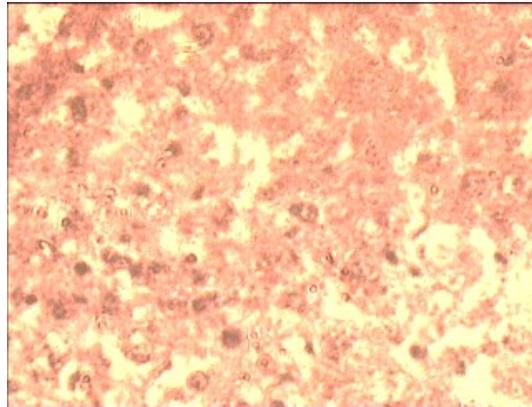


Figure 03 Standard drug treated liver (250 mg/kg body wt)

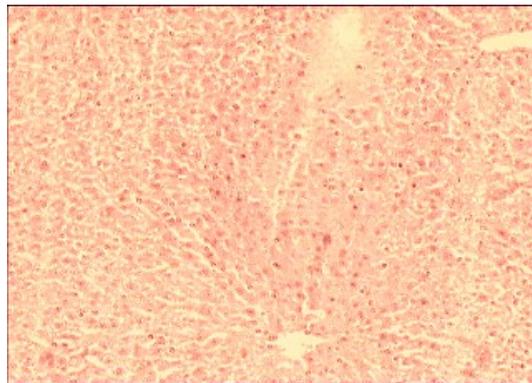


Figure 04 *Vitex negundo* methanolic extract treated liver (200 mg/kg body wt)

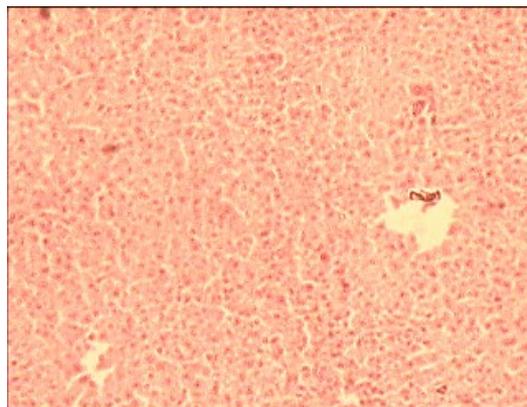


Table 3 Effects of treatment of methanolic extract of *Vitex negundo* on the biochemical parameters of CCl₄ intoxicated rats. (a = P < 0.001, when compared to normal group, b = P < 0.01, c = P < 0.001, when compared to CCl₄ group).

Treatment	Dose	ASAT U/L	ALAT U/L	ALP U/L	Albumin g/L	Total Bilirubin mg/dL	Total Protein g/dL	TGL mg/dL	LDH U/L
Normal	-	82.78 ± 0.537	63.25 ± 13.55	319.70 ± 2.102	3.365 ± 0.295	0.49 ± 0.04	7.466 ± 3.127	73.37 ± 0.22	270.49 ± 0.051
CCl ₄	1ml/kg body wt	180.42 ± 2.04 ^a	119.65 ± 12.18 ^a	579.40 ± 4.604 ^a	1.560 ± 0.104 ^a	0.96 ± 0.02 ^a	4.128 ± 1.42 ^a	29.30 ± 0.12 ^a	530.38 ± 0.04 ^a
Standard + CCl ₄ (1ml/kg body wt)	250 mg/kg body wt	82.48 ± 1.47 ^c	61.55 ± 10.97 ^c	344.25 ± 3.43 ^c	3.312 ± 0.324 ^c	0.45 ± 0.01 ^c	6.544 ± 2.47 ^c	69.25 ± 0.09 ^b	310.20 ± 0.012 ^c
Methanolic extract + CCl ₄ (1ml/kg body wt)	200 mg/kg body wt	85.24 ± 1.29 ^c	63.45 ± 12.41 ^c	350.45 ± 2.394 ^c	3.344 ± 0.208 ^b	0.46 ± 0.013 ^c	6.466 ± 2.61 ^c	66.81 ± 0.17 ^b	318.55 ± 0.104 ^c

Discussion

Liver injuries induced by CCl₄ are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/ or hepatoprotective activities of drugs^[13, 14]. Since the changes associated with CCl₄ induced liver damage are similar to that of acute viral hepatitis^[15], CCl₄ mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl₄ is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450 dependent monooxygenases to form a trichloromethyl radical (CCl₃). The CCl₃ radical alkylates cellular proteins and other macromolecules with simultaneous attack on polyunsaturated fatty acids, in presence of oxygen, to produce lipid peroxides, leading to liver damage^[16]. Thus, antioxidant or free radical generation inhibition is important in protection against CCl₄ induced liver lesions^[17]. Hepatotoxic compounds such as CCl₄ are known to cause marked elevation in serum enzymes and bilirubin levels. It causes marked decrease in TP levels. Silymarin is used as standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes^[18]. To our knowledge, this is the first study which reveals the hepatoprotective effect of methanolic extract of leaves of *Vitex negundo* against CCl₄ induced toxicity in isolated rat hepatocytes, HepG2 cells in culture and in animals, all three models. CCl₄ has been found to induce extensive liver damage within a period of 24 h following intra-peritoneal administration. As a result of this, accumulation of fat in the liver and necrosis in the centrilobular region of the liver occurs. As a consequence, the microsomal enzyme activities are found to decrease and due to lipid peroxidation, the water-soluble enzymes leak into plasma from the liver. It is shown by the significant decrease in triglycerides and proteins in CCl₄ intoxicated rat hepatocytes or animals in the present studies. Treatment with the methanolic extract of *Vitex negundo* exhibited significant restoration of the altered biochemical parameters towards normal in CCl₄ intoxicated rat hepatocytes and in rats. The effect of the methanolic extract at 250 µg/ml was found to be better than that of standard at the same concentration.

Its hepatoprotective effect with *in vivo* studies at 200 mg/kg body weight was comparable to that of standard at 250 mg/kg body weight, positively supported by the histopathology results. The investigation carried out in human liver derived HepG2 cells against CCl₄ induced damage also confirmed the hepatoprotective nature of the methanolic extract of *Vitex negundo*.

Vitex negundo (Verbeneaceae) is a reputed medicinal herb and its parts have been employed as a traditional cure in Asian systems of medicine (Indian, Chinese, Malaysian) for a variety of disease conditions^[19]. A number of pharmacological activities have been attributed to *V. negundo*, such as: analgesic and anti-inflammatory activity^[3], snake venom neutralization activity^[2], hepatoprotective activity^[4], and larvicidal activity^[5]. Alcoholic extract of the seeds of *Vitex negundo* Linn was obtained by cold maceration. A dose of 250 mg/kg of the extract was selected to study the hepatoprotective action against carbon tetrachloride-induced liver damage. The extract was found to be effective in preventing liver damage which was evident by morphological, biochemical and functional parameters^[6]. Hepatoprotective activity of *Vitex negundo* leaf ethanolic extract was investigated against hepatotoxicity produced by administering a combination of three anti-tubercular drugs Isoniazid 7.5 mg/kg, Rifampin 10 mg/kg and pyrazinamide 35 mg/kg. HP effect of *Vitex negundo* leaf ethanolic extract was evident in the doses of 250 and 500 mg/kg as there was a significant control of enzyme levels^[20]. Negundoside, an irridiod glycoside was isolated from leaves of *Vitex negundo*^[19], and its protective mechanism was studied against carbon tetrachloride using human liver cells (HuH-7). The protection afforded by Negundoside seemed to be mediated by activation of cyclic adenosine monophosphate (cAMP) synthesis and inhibition of phospholipases (cPLA2)^[19]. Hence, the hepatoprotective effect observed in the present study may be mainly due to the presence of glycoside in the leaves of *Vitex negundo*. The results from the present study indicate a good correlation between the *in vivo* and *in vitro* studies. In conclusion, the methanolic extract of leaves of *Vitex negundo* merits further investigation in identifying the active constituents responsible for this activity.

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