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# HEPATOPROTECTIVE AND ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACTS OF *NARINGI CRENULATA* (ROXB) NICOLSON AGAINST $CCl_4$ INDUCED HEPATOTOXICITY IN RATS

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## ABSTRACT

The present study was designed to screen and evaluate the hepatoprotective and antioxidant activity of ethanol extracts of leaf and bark of *Naringi crenulata* (Roxb) Nicolson (NCL, NCB) against $CCl_4$ induced hepatotoxicity in rats. Liver functions were assessed by the activities of liver marker enzymes, SGOT, SGPT, ALP, total protein, albumin, globulin, total, conjugated and unconjugated bilirubins. It also exhibited antioxidant activity by showing the increased activity of SOD, CAT, GPx and GRD and decreased in TBARS compared to $CCl_4$ treated groups. Silymarin, a known hepatoprotective drug is used for comparison. The plant extracts were effective in protecting liver against injury induced by $CCl_4$ in rats.

**INTRODUCTION:** Liver is one of the largest chief organs in the human body regulating many important metabolic functions [1]. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [2, 3, 4] and thus, it is an important target of toxicity to xenobiotics, oxidative stress, ethanol and toxic chemicals. The toxins absorbed from the intestinal tract go first to the liver resulting in a variety of liver ailments [5]. Hepatitis and cirrhosis are the two diseases that can badly damage the liver.

Hepatitis is an inflammation of liver caused by certain viruses. Cirrhosis changes the structure of the liver and hence it fails to function properly leading to retention of toxins in the blood. Therefore, damage to the liver inflicted by hepatotoxic agents is of great concern [6]. Drug induced liver toxicity is a common cause of liver injury [7]. Carbon tetrachloride consistently causes liver toxicity, resulting in fatty degeneration, cellular necrosis, fibrosis and cirrhosis [8]. Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for hepatotoxicity due to their inherent distinct physical and chemical properties, leading to diverse pharmacological properties. *Naringi crenulata* (Roxb) Nicolson (Fam-Rutaceae), a tree with green foliage, grows abundantly in different parts of India. The therapeutic effect of the fruits, leaves and roots of this plant in the treatment of different tropical diseases is well documented in the folk medicine [9].

However, no systematic attempts have been made to establish scientific basis of beneficial effects of *Naringi crenulata* leaves and bark extracts. Realising its medicinal properties, the present study aims to investigate the hepatoprotective activity of ethanol extracts of *Naringi crenulata* on carbon tetrachloride ($CCl_4$) induced liver toxicity in rats.

**MATERTIALS AND METHODS:**

**Plant Material:** Leaves and bark of *Naringi crenulata* (Roxb) Nicolson were collected from the Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant was identified with the help of local flora and authenticated in Botanical survey of India, Southern circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology unit, Research Department of Botany, V.O. Chidambaram College, Tuticorin.

**Preparation of plant extracts for Hepatoprotective studies:** The leaf and bark of *Naringi crenulata* were cut into small pieces, washed, shade dried at room temperature and the dried plant materials were powdered in a Wiley mill. The powdered leaf and bark were separately packed in soxhlet apparatus and extracted with ethanol. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts were used for hepatoprotective activity.

**Animals:** Normal healthy male Wistar albino rats (180-240 g) were used for the present investigation. Animals were housed under standard environmental conditions at room temperature (25±2$^{o}$C) and light and dark (12:12h). Rats were fed with standard pellet diet. (Goldmohur brand, MS Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*.

**Acute Toxicity Studies:** Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method) albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study [10]. The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5mg/kg body weight by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 2000 mg /kg body weight.

**Hepatoprotective activity of the extracts against $CCl_4$ toxicity:** In the investigation, the animals were divided into seven groups each consisting of 5 rats.

- Group I- Rats received normal saline was served as a normal control
- Group II- $CCl_4$ hepatic toxicity induced control: Rats received 2.5ml/kg body weight of $CCl_4$ for 7 days.
- Group III- Liver injured rats received ethanol extract of NCL at the dose of 250mg/kg body weight, daily orally for 7 days.
- Group IV- Liver injured rats received ethanol extract of NCL at the dose of 500mg/kg body weight, daily orally for 7 days
- Group V- Liver injured rats received ethanol extract of NCB at the dose of 250mg/kg body weight for 7 days
- Group VI- Liver injured rats received ethanol extract of NCB at the dose of 500mg/kg body weight for 7 days
- Group VII- Liver injured rats received standard drug Silymarin at the dose of 100mg/kg body weight for 7 days.

**Biochemical Analysis:** The animals were sacrificed at the end of experimental period of 7 days by decapitation. Blood was collected, separated by centrifugation at 3000g for 10 minutes. Serum protein [11] and serum albumins were determined quantitatively by colorimetric method using bromocresol green. The total protein minus albumin gives the globulin. Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were measured spectrophotometrically by using the method of Reitman and Frankel [12]. Serum alkaline phosphatase (ALP) was measured by the method of King and Armstrong [13].

Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw [14]. The unconjugated bilirubin concentrations were calculated as the difference between total and conjugated bilirubin concentrations.

**Antioxidant Activity:** For estimating antioxidant activity, Liver homogenates (10% W/V) were prepared in ice cold 10mM tris buffer (pH 7.4). Quantitative estimation of MDA formation (Malondialdehyde) was

done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in 10% liver homogenates by the method of Okhawa [15]. Enzymatic antioxidants, superoxide dismutase [16] (SOD) catalase [17, 18] and non enzymatic antioxidant glutathione peroxidase (GPx) [19] and glutathione reductase [20] (GRD) were also assayed in liver homogenates.

**Statistical Analysis:** The results were expressed as mean ± SEM. The difference among the means has been analysed by one way ANOVA $p<0.05$ and $p<0.01$ were considered as statistical significance using SPSS software.

**RESULTS:** In the present study, the ethanol extracts of leaf and bark of *Naringi crenulata* did not show any sign and symptoms of toxicity and mortality upto 2000mg/kg dose. The effect of ethanol extracts on serum total protein, albumin, globulin, A/G ratio, serum transaminases, alkaline phosphatases in $CCl_4$ intoxicated rats are summarized in **Table 1**. There was significant ($p<0.01$) increase in serum GOT (AST), GPT (ALT) and ALP levels in $CCl_4$ intoxicated group (Group II), compared to the normal control group (Group I). The total protein and albumin were significantly ($p<0.01$ and $p<0.05$) decreased to 6.04 g/dl and 3.56 g/dl in $CCl_4$ intoxicated rats from the levels of 8.51 g/dl and 4.68 g/dl respectively in normal group. Ethanol extracts of NCL and NCB at the dose of 250mg/kg and 500mg/kg orally, significantly decreased the elevated serum marker enzymes and reversed the altered total protein and albumin to almost normal level.

The effect of ethanol extracts of NCL and NCB on total, conjugated and unconjugated bilirubin are shown in **Table 2**. A significant ($p<0.05$) elevation of total, conjugated and unconjugated bilirubin in the serum of $CCl_4$ intoxicated group (Group II) when compared to normal control group (group I). The ethanol extracts of NCL (Group III and IV) and NCB (Group V and VI) at the dose 250mg/kg and 500mg/kg reduced the levels of total, conjugated and unconjugated bilirubin.

The decrease in the concentration of total bilirubin, conjugated bilirubin and unconjugated bilirubin were found to be greater in standard Silymarin (Group VII). The effect is more pronounced in the bark extract. (Group V and VI) (Table 2).

**TABLE 1: EFFECT OF ETHANOL EXTRACTS OF NCL AND NCB ON THE PROTEIN, ALBUMIN, GLOBULIN CONCENTRATION AND ENZYME ACTIVITY OF SERUM GOT, GPT AND ALP IN THE NORMAL, LIVER INJURED AND DRUG TREATED RATS**

| Group | PARAMETERS | | | | | | |
|---|---|---|---|---|---|---|---|
| | T. Protein (mg/dl) | Albumin (g/dl) | Globulin (g/dl) | A/G Ratio | SGOT(U/L) | SGPT(U/L) | ALP(U/L) |
| I | 8.51±0.94 | 4.68±0.34 | 3.83±0.71 | 1.22:1 | 32.56±2.51 | 43.26±2.11 | 164.56±10.21 |
| II | 6.04±0.81** | 3.56±0.12* | 2.48±0.14* | 1.43:1 | 126.53±2.6** | 143.56±5.29** | 269.36±8.4* |
| III | 7.98±0.11 | 4.10±0.16 | 3.88±0.11 | 1.05:1 | 69.53±4.27[a] | 52.26±1.98[a] | 139.54±6.23[a] |
| IV | 8.22±0.13[aa] | 4.98±0.21[a] | 3.24±0.13 | 1.5:1 | 46.53±4.29[aa] | 41.53±1.33[aa] | 129.36±2.6[aa] |
| V | 7.11±0.14 | 4.05±0.18 | 3.06±0.13 | 1.32:1 | 32.76±3.82[aa] | 43.41±1.26[a] | 117.27±10.27[a] |
| VI | 7.82±0.21[a] | 4.81±0.20[a] | 3.51±0.11 | 1.37:1 | 28.11±2.82[aa] | 40.55±1.03[aa] | 122.36±8.92[a] |
| VII | 8.26±0.31[aa] | 4.31±0.12 | 3.95±0.12 | 1.09:1 | 30.59±1.91[aa] | 35.26±1.04[aa] | 131.27±9.34[a] |

Each value is SEM ± 5 individual observations * $p < 0.05$; ** $p<0.01$ Compared with normal control vs liver injured rats [a]: $p < 0.05$; [aa] $p<0.01$ Compared liver injured rats vs drug treated rats

**TABLE 2: EFFECT OF ETHANOL EXTRACTS OF NCL AND NCB ON THE SERUM TOTAL, CONJUGATED AND UNCONJUGATED BILIRUBIN LEVELS IN THE NORMAL, LIVER INJURED AND DRUG TREATED RATS**

| Group | Total Bilirubin (µmol/L) | Conjugated (µmol/L) | Unconjugated (µmol/L) |
|---|---|---|---|
| I | 0.83±0.11 | 0.21±0.01 | 0.62±0.02 |
| II | 2.59±0.34* | 1.46±0.02* | 1.13±0.12 |
| III | 1.14±0.21 | 0.31±0.04[a] | 0.83±0.03 |
| IV | 0.79±0.03[aa] | 0.26±0.06[a] | 0.53±0.03 |
| V | 1.02±0.13 | 0.43±0.01 | 0.59±0.01 |
| VI | 0.86±0.04[aa] | 0.22±0.07[aa] | 0.64±0.04[a] |
| VII | 0.87±0.11[a] | 0.27±0.02[a] | 0.60±0.02[a] |

Each Value is SEM ± 5 individual observations * $p < 0.05$; ** $p<0.01$ Compared with normal control vs liver injured rats [a]: $p < 0.05$; [aa] $p<0.01$ Compared liver injured vs drug treated rats

The effects of ethanol extracts of NCL and NCB on lipid peroxidation (LPO) Superoxide dismutase (SOD), Catalase (CAT) Glutathione peroxidase (GPx) and Glutathione reductase (GRD) activities are shown in **Table 3**.

**Oxidative Stress Markers in Liver Tissue:** The levels of TBARS as an index of lipid peroxidation, a degradative process of membranous lipids, in liver tissue of $CCl_4$ treated rats were significantly ($p<0.05$) elevated when compared to control animals. Lipid peroxidation level was restored towards their normal value by treatment with ethanol extracts of NCL and NCB; indicate prevention of accumulation of lipid peroxidation.

In the present study, glutathione peroxidase, superoxide dismutase and catalase activity were significantly ($p<0.01$) depleted. Similarly, there is significant ($p<0.05$) decrease in the level of glutathione reductase in $CCl_4$ intoxicated rats when compared with those of the animals in control group. Rats treated with ethanol extracts of NCL and NCB at the doses 250 mg/kg and 500 mg/kg significantly decreased the elevated lipid peroxidation levels and restored the altered glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase levels towards the normal levels in a dose dependent manner. The results were well comparable with standard drug (Silymarin) treated group.

**TABLE 3: EFFECT OF ETHANOL EXTRACTS OF NCL AND NCB ON LIVER LPO, SOD, CAT, GPX AND GRD IN THE NORMAL CONTROL, LIVER INJURED AND DRUG TREATED RATS**

| Group | LPO (nM MDA/mg. protein) | SOD (μ/mg protein) | CAT (μ/mg protein) | GPx (μ/mg protein) | GRD (μ/mg protein) |
|---|---|---|---|---|---|
| I | 1.69±0.02 | 4.45±0.12 | 0.53±0.05 | 0.18±0.01 | 2.98±0.04 |
| II | 2.29±0.06* | 1.93±0.11** | 0.17±0.03** | 0.04±0.003** | 1.02±0.36* |
| III | 2.01±0.02 | 2.91±0.16 | 0.39±0.03[aa] | 0.11±0.03*[a] | 2.07±0.02[a] |
| IV | 1.74±0.02[a] | 3.97±0.15[aa] | 0.51±0.03[aa] | 0.18±0.01[a] | 2.89±0.04[a] |
| V | 1.99±0.02[a] | 2.19±1.13 | 0.35±0.04[a] | 0.09±0.23[aa] | 1.97±0.02[a] |
| VI | 2.01±0.05 | 2.99±4.66[a] | 0.40±0.01[aa] | 0.11±0.21[aa] | 2.67±0.03[a] |
| VII | 1.74±0.37[a] | 3.97±4.12[a] | 0.52±0.03[a] | 0.19±0.23[a] | 2.88±0.05[a] |

Each value is SEM ± 5 individual observations; * $p < 0.05$; ** $p<0.01$ Compared with normal control vs liver injured; a $p < 0.05$; aa $p<0.01$ liver injured control vs plant drug treated rats

**DISCUSSION:** $CCl_4$ is one of the most common hepatotoxin, produces an experimental damage that histologically resembles viral hepatitis. Toxicity begins with the metabolism of $CCl_4$, via reductive dehalogenation by cytochrome P450, primarily CYP2E1 metabolites. The following are the key events in hepatotoxicity of $CCl_4$, even leading to cancer [8].

(i) The primary metabolites trichloromethyl and trichloromethylperoxy free radicals are highly reactive and are capable of covalently binding locally to cellular macromolecules, with preference for fatty acids from membrane phospholipids.

(ii) The free radicals initiate lipid peroxidation by attacking polyunsaturated fatty acids in membranes, setting off a free radical chain sequence.

(iii) Lipid peroxidation is known to cause membrane disruption, resulting in the loss of membrane integrity and leakage of microsomal enzymes.

(iv) By-products of lipid peroxidation include reactive aldehydes that can form protein and DNA adducts and may contribute to hepatotoxicity and carcinogenicity respectively.

(v) Natural antioxidants, including glutathione are capable of quenching the lipid peroxidation reaction. When glutathione and other antioxidants are depleted, however, opportunities for lipid peroxidation are enhanced.

(vi) Weakened cellular membranes allow sufficient leakage of calcium into the cytosol to disrupt intracellular calcium homeostasis.

(vii) High calcium levels in the cytosol activate calcium- dependent proteases and phospholipases that further increase the breakdown of the membranes. Similarly, the increase in intracellular calcium can activate endonucleases that can cause chromosomal damage and also contribute to cell death.

(viii) Sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. The increase in cell division coinciding with the increase in frequency of genetic damage can overwhelm DNA repair mechanisms, resulting in an increase in mutagenic frequency and cancer.

The proposed primary mode of action for carbon tetrachloride is shown in **Figure 1**.

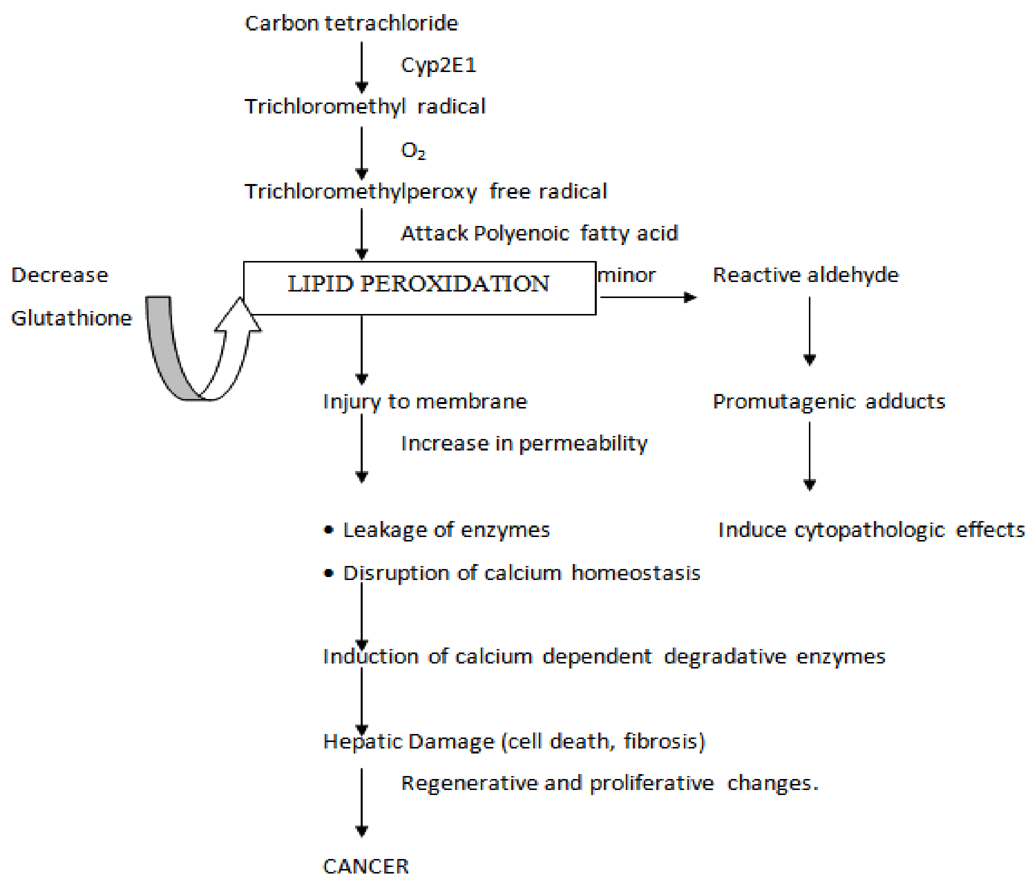


**FIG. 1: PROPOSED PRIMARY MODE OF ACTION FOR CARBON TETRACHLORIDE**

Numerous studies show that metabolism of carbon tetrachloride is required for toxicity. The initial step of carbon tetrachloride metabolism is reductive dehalogenation by cytochrome P450, primarily CYP2E1. Studies using CYP450 inhibitors (eg. 2-diethylaminoethyl 2, 2-diphenyl valerate hydrochloride [SKF – 525A], Silymarin, allylisopropylacetamide) have shown that these compounds inhibit metabolism of Carbon tetrachloride and prevent Carbon tetrachloride induced liver damage [21].

In the present study, there is significant hepatic damage in $CCl_4$ intoxicated rats as shown by the increase in the levels of serum markers AST, ALT and ALP and bilirubin level has been attributed to the damage structural integrity of liver, because they are cytoplasmic in location and released into circulation after cellular damages indicating development of hepatotoxicity [22].

Administration of rats with ethanol extracts of NCL and NCB significantly restored towards their normal value. The normalization of serum markers by ethanol extracts of NCL and NCB suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against $CCl_4$ induced leakage of marker enzymes into the circulation. The above changes can be considered as an expression of the functional improvement of hepatocytes.

Alkaline phosphatase, a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins and alkaloids. The alkaline phosphatase is the prototype of these enzymes that reflects the pathological alteration in biliary flow [23]. Total bilirubin, a by-product of the breakdown of red blood cells in the liver, bilirubin is a good indicator of liver function.

High levels will cause (icterus) jaundice and are indicative of damage to the liver and bile duct [24]. The ethanol extracts of NCL and NCB induced suppression of the increased ALP activity with the concurrent depletion of raised bilirubins suggests the possibility of the extract to have ability to stabilize biliary dysfunction in rat liver during hepatic injury by $CCl_4$.

The reduction of the bilirubins levels by the NCL and NCB extracts also suggest that, the extracts may activate the constitutive androstane receptor (CAR) which is a key regulator in bilirubin clearance in liver [25]. The administration of $CCl_4$ alone may adversely interfere with protein metabolism probably by inhibiting the synthesis of proteins. Administration of ethanol extracts of NCL and NCB significantly reversed these changes by increasing the protein synthesis, thus indicating the protective effect over liver and improvement in the functional efficiency.

Lipid peroxidation has been postulated to the destructive process of liver injury due to $CCl_4$ administration. In the present study, the increase in TBARS (MDA) level in liver induced by $CCl_4$ suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. A massive decrease in lipid peroxidation in liver tissue of plant extracts treated groups indicates that *Naringi crenulata* possess antioxidant properties.

In the present investigations, $CCl_4$ intoxicated rats decreased the content of GPX and GRD in liver, whereas treatment with ethanol extracts of NCL and NCB able to reverse such effects. Superoxide dismutase (SOD) is a key defense enzyme and catalyses the dismutation of superoxide anions. Catalyse (CAT) is a haemoprotein that catalyses the reduction of $H_2O_2$. It converts harmful hydrogen peroxide into water and oxygen and protects the tissues from highly reactive hydroxyl radicals [26].

The reduction in the activity of this enzyme may results in number of deleterious effect due to accumulation of highly toxic, metabolites and $H_2O_2$ on $CCl_4$ administration, which can induce oxidative stress in cells. Administration of ethanol extracts of NCL and NCB, increases the activities of catalase in animals to prevent the accumulation of excessive free radical and protects the liver from $CCl_4$ intoxication. Decrease in SOD activity can result in the removal of superoxide anions that may inactivate SOD thereby cause an inactivation of $H_2O_2$ scavenging enzymes. Administration of ethanol extracts of NCL and NCB prevent effectively the decrease in SOD and CAT activities, which may be directly correlated to scavenging or neutralizing of radicals. The plant extracts of NCL and NCB have greatest ability to reduce oxidative stress by increasing glutathione level and preventing lipid peroxidation.

From our results, it can be concluded that decrease levels of SOD, CAT, GPx, GRD, total protein and albumin and increased serum markers SGOT, SGPT, ALP and lipid peroxidation level in $CCl_4$ treated rats was due to hepatocellular damage. Extracts of leaf and bark of *Naringi crenulata* afforded protection from hepatotoxicity. Both NCL and NCB plant extracts contain antioxidants and hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress.

Thus, the results confirm the hepatoprotective effect of *Naringi crenulata*. The results reveal that the bark of *Naringi crenulata* has shown most pronounced hepatoprotective effect. Further characterization and purification of the individual component in this plant is suggested in formulating the strategy of treatment.

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