# Evaluation of Antioxidant Activity of *Cordia Subcordata* Lam. Against Carbon Tetrachloride ($CCl_4$) Induced Erythrocyte Damage In Rats.

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## Summary

*Cordia subcordata* Lam. a member of the Boraginaceae family, is used in folk medicine because of its treat the hepatic infections, cirrhosis of the liver and inflammation of the lymph nodes. To investigate the antioxidant activity of the ethanol extract of *Cordia subcordata* Lam. (EECS) was investigated in rats with carbon tetrachloride ($CCl_4$) induced erythrocyte damage. Simultaneous administration of the EECS (200 and 400 mg/kg body weight/day i.p) with carbon tetrachloride (1ml/kg of body weight) to rats for alternate days of two weeks protected the loss of functional integrity and membrane lipid alteration in red blood cells induced by oxidative stress. EECS inhibited the accumulation of lipid peroxidation products in the plasma as well as maintained the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase. The EECS further had the ability to decrease the membrane fluidity induced by carbon tetrachloride. It can therefore be suggested that the EECS possess an erythrocyte protective activity against drug induced oxidative stress. These findings also provide a rationale for further studies on isolation of active principles and its pharmacological evaluation.

**Keywords:** Antioxidant enzymes, Carbon tetrachloride, Lipid peroxidation. *Cordia subcordata,* EECS, Erythrocyte Damage.

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## Introduction

Serious attention is now paid to the cytotoxicity of active oxygen/free radicals as the cause of various pathological conditions. Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS), which are the harmful byproducts generated during normal cell aerobic respiration [1].It is widely accepted that antioxidants are radical scavengers, which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neuro-degenertion, Parkinson's diseases, mongolism, ageing process and perhaps dementias [2].

Free radicals such as hydroxy radicals, superoxide anion radicals and singlet oxygens are agents that attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation, a decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation [3]. It is thought that, if the in vivo activity of enzymes or scavengers is not high enough to inhibit these radicals, various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or accelerated aging may result [4].

Lipid peroxidation is also strongly associated with aging and carcinogenesis [5]. However, living systems are protected from active oxygen species by enzymes such as superoxide dismutase, glutathione peroxidase and catalase. These living systems have also been reported to receive non-enzymatic protection by endogenous antioxidants such as α-tocopherol, ascorbic acid, β-carotene, and uric acid [6].

Generally, food antioxidants act as reducing agents, reversing oxidation by donating electrons and hydrogen ions. There is a worldwide trend toward the use of natural antioxidants. The commercial development of plants as sources of antioxidants to enhance health and food preservation is of current interest [7].

***Cordia subcordata* Lam**. Family: Boraginaceae) is a medium-sized spreading tree to 12m tall with grayish grooved flaking bark. Leaves alternate, petiolate, the petiole about half as long as blade, broadly ovate and entire, often wavy-margined, the apex obtuse to short-pointed, base rounded, the blade up to 20 cm long. The seeds float and are highly resistant to salt water, thus the species is common in coastal areas. In Tahiti, the leaves are used in remedies for bronchitis and asthma where the leaves probably act as a purgative. The plant is also used in the treatment of hepatic infections, cirrhosis of the liver and inflammation of the lymph nodes. It is also used to treat albumin present in the urine. Cook Islanders use the leaves in remedies for abdominal swellings and urinary tract infections [8-10]. Therefore, to justify the traditional claims the present study was undertaken to find out if ethanol extract of *Cordia subcordata* Lam. leaves demonstrates the antioxidant activity against $CCl_4$ induced rats models of erythrocyte damage using lipid peroxidation and the antioxidants superoxide dismutase (SOD) and catalase as biomarkers. Hence, the present study was designed to verify the claims of the native practitioners.

## Materials and Methods

### Plant collection

The Plant material of *Cordia subcordata* Lam. leaves was collected from Tirunelveli District, in the Month of August 2008. The plant was authenticated by Dr.V.Chelladurai, Research Officer Botany. C.C.R.A.S., Govt. of India. The voucher specimen (CHE-SA-CS-08) of the plant was deposited at the college for further reference.

### Preparation of plant extract

The leaves of *Cordia subcordata* Lam. were dried in shade, separated and made to dry powder. It was then passed through the 40 mesh sieve. A weighed quantity (80gm) of the powder was subjected to continuous hot extraction in Soxhlet Apparatus. The extract was evaporated under reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample. Percentage yield of ethanolic extract of *Cordia subcordata* was found to be 16.5 % w/w.

### Preliminary phytochemical screening

The phytochemical examination of ethanolic (90%) extract of *Cordia subcordata* Lam. leaves was performed by the standard methods [11]**.**

### Animals used

Male albino rats (150-220g) were obtained from the animal house in C.L. Baid Metha College of Pharmacy, Chennai. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. The animals were fed with standard pellet feed (Hindustan Lever Limited., Bangalore) and water was given *ad libitum*. Ethical committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Ref No. IAEC / XIII / 012 / CLBCP / 2008 - 2009).

### Acute Toxicity Study

The acute toxicity of 90% ehanolic extract of *Cordia subcordata* was determined as per the OECD guideline no. 423 (Acute Toxic Class Method). It was observed that the test extract was not mortal even at 2000mg/kg dose. Hence, $1/10^{th}$ (200mg/kg) and $1/5^{th}$ (400mg/kg) of this dose were selected for further study [**12].**

### Experimental design:

Body weight of animals was recorded and then they were divided into 6 groups of 6 rats each Propylene glycol (PG) was used as a carrier of EECS extracts (200 and 400 mg/kg body weight/day) as well as for carbon tetrachloride (1 ml/kg body weight), administered intraperitoneally alternate days for 14 days. The following experimental groups were used: Group I, II, III, IV, V and VI were received distilled water + PG (Normal control), EECS 200mg/kg + PG (Herb control), EECS 400mg/kg + PG (Herb control),

Carbon tetrachloride in PG, EECS 200mg/kg + carbon tetrachloride in PG, EECS 400mg/kg + carbon tetrachloride, in PG respectively. On the 15$^{th}$ day rats were kept fasting for 12 hours and sacrificed by cervical dislocation. Blood was collected from the jugular vein into tubes containing heparin, centrifuged at 3000 rpm for 15 min and the resulting buffy coat removed. The packed cells were washed three times with physiological saline (0.9% NaCl), lysed by suspending them in cold distilled water, and then centrifuged at 7000 rpm for 30 min. The resulting pellet contained the erythrocyte membrane and the supernatant represented the haemolysate.

**Biochemical estimation**

Plasma resulting from the initial centrifugation was used for measuring lipid peroxidation following the method of Gutteridge and Wilkins [13] while the haemolysate was used for the estimation of superoxide dismutase [14] and catalase [15] activities. Lipids from the erythrocyte membrane were extracted using the method of Folch et al [16]. The concentration of cholesterol and phospholipids were determined using previously established methods [17,18]. The cholesterol/phospholipid ratio was then calculated.

**Statistical analysis**

The data were expressed as mean ± standard error mean (S.E.M).The Significance of differences among the group was assessed using one way and multiple way analysis of variance (ANOVA). The test followed by Dunnett's test p values less than 0.05 were considered as significance.

## Results

The results of preliminary phytochemical screening of the ethanoloic extract of *Cordia subcordata Lam.* revealed that presence of alkaloids, flavonoids, carbohydrates, glycosides, tannins, terpeniods and absence of saponins and steroids.

Table-1 shows the effect of EECS on carbon tetrachloride induced oxidative stress. Treatments with the extracts significantly (P <0.01) prevented the accumulation of lipid peroxidation products in the plasma. Intoxication of the rats with carbon tetrachloride also led to significant increases in superoxide dismutase and catalase activities, while simultaneous administration of carbon tetrachloride with the EECS significantly (P <0.01) decreased these activities.

Intoxication with carbon tetrachloride causes an increase in membrane cholesterol, a decrease in membrane phospholipid and a subsequent increase in the cholesterol to phospholipid ratio. At the doses of EECS 200 & 400 significantly (P <0.01) decreases the cholesterol, phospholipids and cholesterol/phospholipid ratio also (Table 2).

**Table 1:** The effects of *EECS* on lipid peroxidation products and primary antioxidant enzymes of the erythrocytes of carbon tetrachloride -intoxicated rats.

| Group | Design of treatments | Lipid peroxidation x $10^{-6}$ (units) | Enzyme activities (Units/mg protein) | |
|---|---|---|---|---|
| | | | Superoxide dismutase | Catalase |
| I | Control, PG | 0.28 ± 0.03 | 192.6 ± 3.3 | 1.9 ± 0.2 |
| II | EECS 200mg/kg + PG | 0.27 ± 0.05 | 194.2 ± 2.7 | 1.8 ± 0.1 |
| III | EECS 400mg/kg + PG | 0.28 ± 0.02 | 191.7 ± 3.2 | 1.8 ± 0.2 |
| IV | $CCl_4$ + PG | 0.48 ± 0.03$^{a}$ | 274.4 ± 1.7$^{a}$ | 4.7 ± 0.6$^{a}$ |
| V | $CCl_4$ + EECS 200mg/kg | 0.38 ± 0.04$^{*b}$ | 229.1 ± 2.3$^{*b}$ | 3.2± 0.2$^{*b}$ |
| VI | $CCl_4$ + EECS 400mg/kg | 0.32 ± 0.06$^{**b}$ | 216.0 ± 1.8$^{**b}$ | 2.3 ± 0.6$^{**b}$ |

PG=Propylene glycol; Carbon tetrachloride= $CCl_4$; EECS=Ethanol extract of *Cordia subcordata Lam.* Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test. **a** means Comparison between Group IV Vs Group I, II & III. **b** means Comparison between Group V and VI Vs Group I, II, III& IV. *p<0.05;** p<0.01; ns-non significant.

**Table 2**: Effect of *EECS* on erythrocyte membrane lipids and cholesterol/phospholipid ratio of carbon tetrachloride -intoxicated rats.

| Group | Design of treatments | Cholesterol (mg/100µl) | Phospholipid (mg/100µl) | Cholesterol /Phospholipid |
|---|---|---|---|---|
| I | Control, PG | 0.65 ± 0.03 | 1.09 ± 0.04 | 0.60 ± 0.04 |
| II | EECS 200mg/kg + PG | 0.64 ± 0.02 | 1.10 ± 0.03 | 0.61 ± 0.02 |
| III | EECS 400mg/kg + PG | 0.65 ± 0.04 | 1.08 ± 0.04 | 0.59 ± 0.05 |
| IV | $CCl_4$ + PG | 0.84 ± 0.05$^{a}$ | 0.86 ± 0.$^{02a}$ | 0.99 ± 0.$^{03a}$ |
| V | $CCl_4$ + EECS 200mg/kg | 0.73 ± 0.02$^{*b}$ | 0.95 ± 0.03$^{*b}$ | 0. 77 ± 0.02$^{*b}$ |
| VI | $CCl_4$ + EECS 400mg/kg, | 0.68 ± 0.03$^{**b}$ | 0.99 ±0.02$^{**b}$ | 0.70 ± 0.04$^{**b}$ |

PG=Propylene glycol; Carbon tetrachloride= $CCl_4$; EECS=Ethanol extract of *Cordia subcordata Lam.*

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test. **a** means Comparison between Group IV Vs Group I, II & III. **b** means Comparison between Group V and VI Vs Group I, II, III& IV. *p<0.05;** p<0.01; ns-non significant

**Discussion and conclusion**

The results obtained in this study indicate the rigidity of the membranes. Administration of EECS prevented changes in membrane lipids as well as those in membrane fluidity. It is well recognized that free radicals are critically involved in various pathological conditions such as cancer, cardiovascular disorders, arthritis, inflammation and liver diseases [19]. Under normal physiological conditions low concentrations of lipid peroxidation products are found in tissues and cells. In the presence of oxidative stress more lipid peroxidation products are formed due to cell damage. Cellular antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase normally challenge oxidative stress. In this study, carbon tetrachloride damage to erythrocytes was confirmed by the increases in lipid peroxidation products, superoxide dismutase and catalase activities, and decreases in membrane fluidity. The increased superoxide dismutase activity resulted in the accumulation of hydrogen peroxide, which stimulated increases in catalase activity. Pre-treatment of experimental animals with the EECS exhibited an improved free radical scavenging resulting in decreased activities of superoxide dismutase and catalase, and the concentration of lipid peroxidation products towards normal.

The cumulative effect of carbon tetrachloride resulted in increases in erythrocyte membrane peroxidation, which may also lead to hemolytic changes. It has been shown that micro-viscosity of a membrane increases markedly with increases in cholesterol to phospholipid ratio thus leading to cellular rigidity [20]. Intoxication of experimental animals with carbon tetrachloride altered membrane structure and function as shown by the increases in cholesterol and subsequent decreases in phospholipid concentrations, hence increased cholesterol to phospholipid ratio. Cooper et al [21] reported that alteration of bio-membrane lipid profile disturbs its fluidity, permeability, activity of associated enzymes and transport system. Thus *Cordia subcordata* plays a role in peroxidation by inhibiting the free radical attack on bio-membranes.

The results of phytochemical screening of the EECS revealed that presence of flavonoids and terpeniods. The terpenoids have been reported to protect lipids, blood and body fluids against the attack of reactive oxygen species like superoxide, peroxide and hydroxyl radicals. In experimental studies, terpenoids have prevented the occurrence of cancer in many tissues including, breast, colon, stomach, prostate, pancreas, liver and skin [22-25]. These include free radicals such as hydroxyl radicals, aqueous peroxyl radicals, and superoxide anion. Moreover, Flavonoids also reported to have antioxidant activity [27]. The presence of terpenoids, and flavonoids in *Cordia subcordata* might be responsible for their observed antioxidant activity.

Since reactive oxygen species are involved in stress and pathogenesis of cancer, diabetes mellitus, atherosclerosis, and dementia, the use of this plant may be beneficial in preventing initiation or progress of such disorders. Efforts are in progress in our laboratory to isolate and purify the active principle involved in the antioxidative efficacy of this medicinal plant.

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