Ravichandran S & Panneerselvam P. / *International Journal of Preclinical & Pharmaceutical Research.* 2014; 5(1): 19-24.

e-ISSN 2249-7552
Print ISSN 2229-7502

International Journal of
Preclinical & Pharmaceutical Research

Journal homepage: www.preclinicaljournal.com

INVIVO ANTIOXIDANT PROPERTIES OF COMBINED EXTRACTS OF *CARDIOSPERMUM HALICACABUM L.* AND *DELONIX ELATA L.* LEAVES ON CCL_4 INDUCED ERYTHROCYTE DAMAGE IN RATS

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ABSTRACT

The present study was estimated the antioxidant properties of combined petroleum ether extracts of *Cardiospermum halicacabum L.* and *Delonix elata L.* leaves (CPCD) by CCl_4 induced rats models of erythrocyte damage in rats. Vehicle and extract treatments once daily for 14days, but CCl_4 (1 ml/kg of body weight, i.p), alternate days for 14 days. On the 15th day all group of animals were kept fasting for 12 h and sacrificed by cervical dislocation. Blood was collected and estimated of lipid peroxidation, superoxide dismutase, catalase, Glutathione Reductase and Glutathione peroxidase [17] activities. The present study suggested that oxidative stress and lipid peroxidation rise might occur after CCl_4 administration. In present study results showed that CPCD significantly decreased lipid peroxidation and increased superoxide dismutase, catalase, Glutathione Reductase and Glutathione peroxidase. The presence of flavonoids and polyphenols in petroleum ether extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. might be responsible for their observed antioxidant activity. Since reactive oxygen species and free radicals were involved in oxidative stress and pathogenesis of cancer, diabetes mellitus, atherosclerosis, and inflammation, the use of these plants may be beneficial in preventing initiation or progress of many diseases.

Key Words: *Cardiospermum halicacabum L.*, *Delonix elata L.*, Antioxidant, Erythrocyte Damage.

INTRODUCTION

Free radicals (hydroxy radicals, superoxide anion radicals and singlet oxygens) are agents that attack the unsaturated fatty acids in the bio membranes of the cell 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 [1,2]. It is thought that, if the in vivo activity of enzymes or scavengers is not high enough to inhibit these radicals, accelerated the various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or aging may result [3].

Cardiospermum halicacabum L. is belongs to Family**:** Sapindaceae. It has been used in Indian traditional medicine for a long time in the treatment of rheumatism, stiffness of the limbs and snakebite [4,5]. Experimental pharmacological studies have shown the analgesic, anti-inflammatory, antipyretic, antimalarial, antioxidant activity, and anti-ulcer and vasodepressant activities [6-10].

Delonix elata L. (Family:Fabaceae) is a deciduous tree about 2.5-15 m tall, with a spreading, rather rounded crown, crooked poor stem form and drooping branches. The plant is traditionally used for the treatment of abdominal pains, rheumatism and flatulence. The stem bark of this plant is considered as good febrifuge and is

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much appreciated as an antiperiodic & anti-inflammatory [11].

There are no reports found for prove synergistic effect of combined extracts of *Cardiospermum halicacabum* L. and *Delonix elata* L. leaves. Therefore the present study was estimated the antioxidant properties of combined petroleum ether extracts of *Cardiospermum halicacabum L.* and *Delonix elata L.* leaves (CPCD) by CCl_4 induced erythrocyte damage in rats.

MATERIALS AND METHODS

Plant collection

The leaves of *Cardiospermum halicacabum* L. and *Delonix elata* L. used for investigation and it was collected from Tirunelveli District, in the Month of August 2010. The plant was authenticated by Dr.V.Chelladurai, Research Officer Botany. C.C.R.A.S., Govt. of India.

Preparation of extracts

The leaves of plants were dried in shade, separated and made to dry powder. It was then passed through the 40 mesh sieve. A weighed quantity (100gm) of the each powder was subjected to continuous hot extraction in separate Soxhlet apparatus. The extract was evaporated under reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample. The petroleum ether extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. yielded thick green semi-solid residues. Percentage yield of *Cardiospermum halicacabum* L. and *Delonix elata* L. was found to be 2.5% and 2.3% w/w.

Preliminary phytochemical screening

The phytochemical qualitative chemical composition of petroleum ether extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. using commonly employed precipitation and coloration to identify the major natural chemical groups such as steroids, reducing sugars, alkaloids, phenolic compounds, saponins, tannins, flavonoids, amino acids and glycosides were performed by the standard methods [12]. General reactions in theseanalysis revealed the presence or absence of these compounds in the crude extracts tested.

Animals used

Wistar strain of albino rats (150-200g) 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 (Reference No: IAEC/XII/011/CLBMCP/2010-2011.

Experimental design

Body weight of animals was recorded and then they were divided into 5 groups of 6 rats each. The following experimental groups were used:

Group I - Received vehicle [Normal control] 1% w/v SCMC, 1ml/100 g

Group II - CCl_4 (1 ml/kg of body weight), i.p [Negative Control]

Group III - Received combined Petroleum ether extracts of *Cardiospermum halicacabum* L. and *Delonix elata* L. (CPCD) (400mg/kg body weight p.o) suspended in 1% w/v SCMC + CCl_4 (1 ml/kg of body weight), i.p

Vehicle and extract treatments once daily for 14days, but CCl_4 (1 ml/kg of body weight, i.p), alternate days for 14 days.

On the 15^{th} day all group of animals were kept fasting for 12 h and sacrificed by cervical dislocation. Blood was collected from the jugular vein into tubes containing heparin (anticoagulant), 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 7000rpm 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], catalase [15], Glutathione Reductase [16] and Glutathione peroxidase [17] activities. The concentration of cholesterol and phospholipids were determined standard methods [18]. The cholesterol/phospholipid ratio was then calculated.

RESULTS

Effect of oral dose of CPCD on lipid peroxidation of the erythrocytes of carbon tetrachloride -intoxicated rats

Treatments with the CPCD significantly ($P < 0.01$) prevented the accumulation of lipid peroxidation products in the plasma. Oral dose of CPCD (400mg) treated group showed more significant effect than control group**.** But carbon tetrachloride treated rats clearly indicates that the CCl_4 increases the accumulation of lipid peroxidation (50.33±0.9189) in plasma (Table 1).

Effect of oral dose of CPCD on Superoxide dismutase (SOD) of the erythrocytes of carbon tetrachloride - intoxicated rats

Superoxide dismutase activity was evaluated to estimate endogenous defenses against superoxide anions. In control animals, normal SOD activity was 189.33±1.085 Unit /mg of protein. In contrast, a significantly increased

the SOD was seen in CCl_4 (1 ml/kg of body weight), i.p treated rats (257.67±3.630). Administration of oral dose of CPCD significantly ($p<0.01$) decreased SOD activities than control group (Table 2).

Effect of oral dose of CPCD on catalase of the erythrocytes of carbon tetrachloride -intoxicated rats

Intoxication of the rats with carbon tetrachloride also led to significant increases in catalase activity while simultaneous administration of carbon tetrachloride with oral dose of CPCD significantly ($P<0.01$) decreased these activities than control group (Table 3).

Effect of oral dose of CPCD on Glutathione reductase & Glutathione peroxidase of the erythrocytes of carbon tetrachloride-intoxicated rats

Intoxication with carbon tetrachloride causes inversely decreases the glutathione reductase & glutathione peroxidase in erythrocyte membrane fluidity. Administration of oral dose of CPCD (400mg/kg, p.o) increased glutathione reductase & glutathione peroxidase activities than control group (Table 4 & 5).

Effect of oral dose of CPCD on cholesterol and phospholipids

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. Administration of oral (CPCD 400mg/kg, p.o) significantly ($P<0.01$) decreases the cholesterol and phospholipids (Table 6,7&8).

Table 1. Effect of oral dose of CPCD on lipid peroxidation of the erythrocytes of carbon tetrachloride -intoxicated rat

Group	Design of treatments	Lipid peroxidation x 10^{-6} (units)
I	Vehicle 1%w/v SCMC,1ml/100g p.o	26±0.5774**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	50.33±0.9189
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	36.50±0.4282**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III, IV & V. *p<0.05;** p<0.01;

Table 2. Effect of oral dose of CPCD on Superoxide Dismutase of the erythrocytes of carbon tetrachloride -intoxicated rat

Group	Design of treatments	Superoxide Dismutase Units/mg protein
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	189.33±1.085**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	257.67±3.630
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	233.17±0.8724**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III, IV & V. *p<0.05;** p<0.01;

Table 3. Effect of oral dose of CPCD on Catalase of the erythrocytes of carbon tetrachloride -intoxicated rat

Group	Design of treatments	Catalase Units/mg protein
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	1.67±0.0494**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	4.52±0.0601
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	3.42±0.0601**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test.**a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

Table 4. Effect of oral dose of CPCD on Glutathione Reductase of the erythrocytes of carbon tetrachloride -intoxicated rat

Group	Design of treatments	Glutathione Reductase GSSG utilized/min/mg protein
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	34.17±0.9458**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	21.83±0.7032
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	24.83±0.3073**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

Table 5. Effect of oral dose of CPCD on Glutathione Peroxidase of the erythrocytes of carbon tetrachloride -intoxicated rat

Group	Design of treatments	Glutathione Peroxidase $NADP^+$ / min/ mg protein
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	25.33±0.4944**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	15.50±0.4282
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	21.83±0.4773**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

Table 6.Effect of oral dose of CPCD on erythrocyte membrane Cholesterol of carbon tetrachloride -intoxicated rats

Group	Design of treatments	Cholesterol (mg/100µl)
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	0.66±0.0118**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	0.91±0.0117
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	0.85±0.0096**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test.**a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

Table 7. Effect of oral dose of CPCD on erythrocyte membrane Phospholipid of carbon tetrachloride -intoxicated rats

Group	Design of treatments	Phospholipid (mg/100µl)
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	1.12±0.0099**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	0.86±0.0071
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	0.91±0.0082**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

Table 8. Effect of oral dose of CPCD on erythrocyte membrane Cholesterol/Phospholipid ratio of carbon tetrachloride -intoxicated rats

Group	Design of treatments	Cholesterol/Phospholipid
I	Vehicle 1%w/v SCMC, 1ml/100g p.o	0.59±0.02**[a]
II	CCl_4 (1 ml/kg of body weight), i.p	1.06±0.01
III	CPCD (400mg/kg b.w, *p.o*) + CCl_4	0.93±0.02**[b]

Values are expressed as mean ± SEM of six observations. Statistical significant test for comparison was done by ANOVA, followed by Dunnett's test. **a** - Comparison between Group I Vs Group II. **b** - Comparison between Group II Vs Group III. *p<0.05;** p<0.01;

DISCUSSION AND CONCLUSION

The results of *invivo* antioxidant activity of CPCD clearly indicated that the rigidity of the membranes after administration of oral (400mg/kg, p.o). Oral dose of CPCD prevented changes in membrane phospholipids as well as those in membrane fluidity. Many of the authors proved free radicals are critically involved in various pathological conditions such as cancer, arthritis, inflammation and liver diseases [19].

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS) may provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemicals or by other means. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and some other machanisms [20]. The present study, carbon tetrachloride intoxication damage to erythrocytes was confirmed by the elevation of lipid peroxidation, superoxide dismutase and catalase activities, and inversely decreases the glutathione reductase & glutathione peroxidase in erythrocyte 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 oral dose of CPCD exhibited an improved free radical scavenging resulting in decreased activities of superoxide dismutase and catalase, and the concentration of lipid peroxidation products towards normal.

In normal tissues and cells, the concentrations of lipid peroxidation are found to be less. In the presence of oxidative stress more lipid peroxidation products are formed due to cell damage. Previous studies concluded that alteration of glutathione peroxidase and glutathione

reductase activity in erythrocytes was inversely correlated with intensity of lipid peroxidation. The failure of H_2O_2 detoxification due to decrease in glutathione peroxidase and glutathione reductase activity. H_2O_2 accumulated in erythrocyte cells iron ions present may undergo Fenton's reaction in which hydroxy radicals are produced. These reactive oxygen species participate in lipid peroxidation processes [21-23]. Increases in lipid peroxidation in the present study were dependent on decrease in glutathione peroxidase & glutathione reductase activity.

The present study suggested that oxidative stress and lipid peroxidation rise might occur after CCl_4 administration. In present study results showed that CPCD significantly decreased lipid peroxidation and increased glutathione peroxidase & glutathione reductase. Participation of oxygen free radicals and oxidative stress in carbon tetrachloride (CCl_4) induced erythrocyte damage may indirectly be confirmed by antioxidant activity of CPCD.

Previous authors revealed [24] that alteration of bio-membrane lipid profile disturbs its permeability, fluidity, activity of associated enzymes and transport system. The cumulative effect of CCl_4 intoxication resulted that microviscosity of a membrane increases markedly with increases in cholesterol to phospholipid ratio thus leading to cellular rigidity [25]. 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. Thus CPCD plays a key role in peroxidation by inhibiting the free radical attack on bio-membranes.

The results of phytochemical screening of the petroleum ether extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. revealed that presence of flavonoids and polyphenols. The presence of flavonoids has been reported to protect lipids, blood and body fluids against the attack of reactive oxygen species like superoxide, peroxide and hydroxyl radicals [26-28]. Polyphenols also one of the very important plant constituents because of their free radical scavenging activity due to their hydroxyl groups [29,30].

The presence of flavonoids and polyphenols in petroleum ether extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. might be responsible for their observed antioxidant activity. Since reactive oxygen species and free radicals were involved in oxidative stress and pathogenesis of cancer, diabetes mellitus, atherosclerosis, and inflammation, the use of these plants may be beneficial in preventing initiation or progress of many diseases. Further studies regarding the isolation and characterization of the active principles responsible for antioxidant activity is currently under progress.

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