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Enzymatic and non-enzymatic antioxidant activity of *Pergularia tomentosa* against carbon tetra chloride induced hepatic damage in Wistar albino rats

Vinoth Kumar Pothagar[1*], Malliga Elangovan[2] and Raja Sekar Srinivasan[3]

[1]Department of Microbiology, Southwest Medical University, Luzhou city, 646000, PR China
[2]Department of Biochemistry, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India
[3]Department of Microbiology, Sribharathi Arts and Science College, Kaikkurichy, Pudukottai, Tamil Nadu, India

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ABSTRACT

Many oxidative stress related disease are due to accumulation of free radicals in the body which causes cell injury. In this study, the enzymatic antioxidants (Superoxide dismutase, Catalase, Glutathione-s-transferase, Glutathione peroxidase, Ascorbate oxidase and Polyphenoloxidase) and non-enzymatic antioxidants (Total reduced glutathione and Vitamin C) activities were determined using Carbon tetrachloride (CCl_4) rat liver as experimental model. The ethanol and chloroform extract showed noticeable increases in enzymatic antioxidant and non- enzymatic antioxidant and thus capability to scavenge the free radicals and protect against oxidative stress causing diseases. Thus, the present study indicates that the plant may be clinically valuable agent in the prevention of hepatic failure caused by CCl_4 intoxication.

Key Words: *Pergularia tomentosa*, enzymatic antioxidants, non-enzymatic antioxidants.

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Corresponding author: **Vinoth Kumar Pothagar**
e-mail✉*vinothkumarphd@gmail.com*
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INTRODUCTION

Most of the researches are going on worldwide are directed towards the finding of natural antioxidants of plants origins. Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various human ailments because they contain the components of therapeutic value (1). It was evident from the recent studies that large number of herbs products including polyphenolic substances can be considered as the most abundant plant secondary metabolites with highly diversified structures. This source of phytochemicals strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals (2).

When the Reactive Oxygen Species (ROS) were in imbalance, oxidative stress in the human body is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ageing, cardiovascular disease, diabetes mellitus, cancer, immune-suppression, neurodegenerative diseases and other diseases (3). A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. Oxidative process is one of the most important routes for producing free radicals in foods, drugs and even in living systems (4). Antioxidants are the compounds which terminate the attack of free radicals and thus reduce the risk of these disorders (5). The use of natural antioxidants for treating diseases and as food additives has better consumer acceptability and a trend over the use of

the available synthetic products. Many research groups therefore have taken the responsibility of screening and quantification of the antioxidant activities of the medicinal plant (6).

Pergularia tomentosa L. is an evergreen perennial shrub belong to the family: Asclepiadecae and the height of this shrubby plant species is about one meter with peculiar odour (7). This plant is used in folk remedies as an antirheumatic, laxative, abortive and treatment of some skin diseases, asthma and bronchitis (8). The plant has different biological effects such as cytotoxic, antioxidant and antibacterial activities which are due to its cardenolide and flavonoid compounds (9 - 11).

Almost all organisms are protected to some extent by free radical damage with the help of enzymes such as *superoxide dismutase, catalase* and antioxidant compounds viz. ascorbic acid, tocopherol, phenolic acids, polyphenols, flavonoids and glutathione. In last two decades, several medicinal plants have shown such effectiveness through the traditional methods for pharmacological function (12). The present study has been conducted to evaluate the enzymatic antioxidants and non-enzymatic antioxidants activities using Carbon tetrachloride (CCl_4) rat liver as experimental model.

MATERIALS AND METHODS

Plant Extraction

The leaves of the *P.tomentosa* were dried under shade, powdered with a mechanical grinder, and passed through sieve no. 40. The sieved powder was stored in airtight container and kept in room temperature. Dried plant material (500g) was extracted with Soxhlet apparatus using petroleum ether to remove chlorophyll. After defatting, it was dried in hot air oven at 50°C and stored in air-tight bottles. 100gm of powder was extracted by soxhlet apparatus using 400 ml of ethanol (95% V/v) and chloroform solvent carefully for about 72 hours at 58-60^{o} C. The extracts were concentrated under reduced pressure by using rotary vacuum evaporator and dried at -40 ^{o} C in a freeze drier.

Animals

In the present study healthy, matured male albino rats (Wistar strain) were used. Rats weighing 180-230 g were obtained from the Periyar College of Pharmaceutical Sciences, Tiruchirapalli, Tamil Nadu, and India and kept in plastic animal cages with 12 hr light and dark cycle in the institutional animal house. The animals had free access to standard rodent diet and provided water ad *libitum*. After one week of acclimatization the animals were used for the further experiments. The study protocol was approved by the Institutional Animal Ethical Committee for the usage of animals in the experiments was obtained as per the Indian CPCSEA guidelines (Registration Number: 265/CPCSEA)

Acute Toxicity Study

Acute toxicity studies were carried out using Acute Toxic Class Method as per OECD-423 Guidelines. Ethanol and Chloroform leaf extract of *P. tomentosa* were orally administered at a starting dose of 2000 mg/kg body weight to 4 male rats. The animals were observed for mortality and behavioral changes during 48 hours (13).

Experimental Design

A total of 90 animals were randomly divided into 15 groups of six animals each (n=6/ group).

Group I (Control): received only 3% gum acacia.

Group II to IV: received the leaf ethanol extracts of *P. tomentosa* in 50, 100 and 200 mg/kg body weight respectively.

Group V to VII: received the leaf chloroform extracts of *P. tomentosa* in 50, 100 and 200 mg/kg body weight respectively.

Group VIII (Hepatotoxin control): received a single dose of 2mg/kg of CCl_4.

Group IX to XI: were administrated with a single dose of 2mg/kg of CCl_4 and followed by the treatment with leaf ethanol extracts of *P. tomentosa* in 50, 100 and 200 mg/kg body weight respectively.

Group XII to XIV: were administrated with a single dose of 2mg/kg of CCl_4 and followed by the treatment with leaf Chloroform extracts of *P. tomentosa* in 50, 100 and 200 mg/kg body weight respectively.

Group XIV (Hepatoprotective agent control): animals were administered with CCl_4 for 14 days and followed by the treatment with 100 mg/kg body weight of known hepatoprotective agent (Silymarin).

Serum Preparation

On the 15th day, blood was collected from retro-orbital plexus, allowed to clot for 1 h at room temperature and serum was separated by centrifugation at 2500 rpm at 30 °C for 15 min. The serum was then collected and analyzed for various biochemical parameters.

Biochemical Analysis:

Assay of Superoxide Dismutase (SOD)

SOD was assayed according to the method of Das (14). The sample extract (100µl) was incubated with 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) at 30ºC for 5 minutes. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min to 200 W- Philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

Assay of Catalase (CAT)

The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, acid reagent and then the enzyme was added. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein. CAT activity was measured as described by Sinha (15).

Assay of Glutathione Peroxidase (GPX)

Glutathione peroxidase was assayed according to the method of Rotruck (16) with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodiumazide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and then centrifuged; 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The colour developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein.

Assay of Glutathione-S-Transferase (GST)

Glutathione-S-transferase activity using 2, 4 dichloronitrobenzene as substrates was assayed spectrophotometrically according to the procedure of Habig (17). The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM of chlorodinitrobenzene and 20 μl of appropriately diluted plant extract from the different sources. Change in absorbance at 340 nm was followed against a blank containing all reactants excepting enzyme protein, Specific activity was expressed as µmol conjugate formed/min/mg protein

Assay of Ascorbate Oxidase

Assay of ascorbate oxidase activity was carried out essentially as described by Vines and Oberbacher (18). The sample was homogenized (1: 5 (w/v)) with phosphate buffer (0.1 M/ pH 6.5) and centrifuged at 3000 g for 15 min at 50°C. The supernatant obtained was used. To 3.0 ml of the substrate solution (8.8 mg ascorbic acid in 300 ml phosphate buffer, pH 5.6), 0.1 ml of the plant extract was added and the absorbance change at 265 nm was measured for every 30 seconds for a period of 5 minutes. One enzyme unit is equivalent to 0.01 O.D. changes per min.

Assay of Polyphenol Oxidase (PPO)

Assay of Polyphenol oxidase activity was carried out according to the procedure of Sadasivam and Manickam (19). To 2.0 ml of plant extract and 3.0ml of distilled water added and mixed together. 1.0ml of cathecol solution (0.4mg/ml) added to the above solution and the reactants were quickly mixed. The enzyme activity was measured as change in absorbance/min at 490nm.

Estimation of Reduced Glutathione (TRG)

The amount of reduced glutathione in the samples was estimated by the method of Boyne and Ellman (20). 1ml of the sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M

Na_2HPO_4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri sodium citrate). Absorbance was read at 412 nm within 2 minutes. GSH concentration was expressed as nmol/mg protein.

Estimation of Vitamin-C

As per Yen and Chen (21), the assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37ºC for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of µg/mg plant tissue.

Statistical Analysis

All data were expressed as Mean ± Standard deviation of number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 9.0 (SPSS, Cary, NC, USA) and Duncan's multiple range test (22). A value of $p < 0.05$ was considered to indicate a significant difference between groups.

RESULTS AND DISCUSSION

Enzymatic Antioxidants

Both the leaves extracts of *P.tomentosa* (50, 100 and 200 mg/kg) given orally for 14 days showed hepatoprotective activity in CCl4 induced hepatic damage in rats. There was a significant increases in *Superoxide dismutase* activities, *Catalase*, *Glutathione peroxidase* and *Glutathione S transferase* in liver was observed in Group- IX, X, XI, XII, XIII, XIV, XV rats when compared with toxicity group. When compared with group I control rats, Group VIII rats shows decreases in the enzyme activities such as SOD, CAT, GPx and GST. There is no significant difference was observed in group II, III, IV, V, VI, VII when compared with Group I rat (Table-1).

Treated groups are compared with toxicity (CCl_4) control; # values are statistically significant at $P < 0.01$ compared to toxicity (CCl_4) control. Significant increase in *Ascorbate oxidase* and *Polyphenol oxidase* in liver was noticed in group-IX, X, XI, XII, XIII, XIV, XV rats when compared with toxicity group, however group-VIII rats shows reduction in the activities of Ascorbate oxidase and Polyphenol oxidase when compared with group I rats. There is no significant difference was observed in group- II, III, IV, V, VI, and VII when compared with group-I rats (Table-2).

Toxicity (CCl_4) is compared with the normal; * Values are statistically significant at $P < 0.01$ compared to control. Treated groups are compared with toxicity (CCl_4) control; # Values are statistically significant at $P < 0.01$ compared to toxicity (CCl_4) control.

Table No. 1 The level of enzymatic antioxidants such as SOD, CAT, GPx, and GST

S.NO	Groups	SOD	CAT	GPx	GST
1	Normal	18.25±1.57	64.6±7.9	262.28±1.91	294.10±0.28
2.	Ethanol 50 mg/kg	16.32±0.14	68.8±1.7	260.71±0.25	291.12±1.34
3	Ethanol 100 mg/kg	16.22±0.17	65.8±1.6	261.86±0.82	290.01±0.08
4	Ethanol 200 mg/kg	16.98±1.32	66.7±2.4	260.12±1.27	289.21±1.25
5	Chloroform 50 mg/kg	16.84±0.44	66.9±0.7	261.95±3.01	291.22±0.18
6	Chloroform 100 mg/kg	17.20±2.32	65.4±1.3	260.90±1.42	290.62±0.98
7	Chloroform 200 mg/kg	17.15±0.92	66.9±2.1	261.82±1.04	291.91±0.26
8	Carbon tetrachloride (CCl_4) - 2 mg/kg	1.21±0.30*	15.41±2.91*	91.89±1.29*	98.28±0.91*
9	CCl_4 + Ethanol 50 mg/kg	9.27±3.97#	61.6±7.9#	180.83±1.20#	171.14±0.11#
10	CCl_4 + Ethanol 100 mg/kg	10.89±1.25#	62.8±7.9#	184.21±0.22#	172.28±1.02#
11	CCl_4 + Ethanol 200 mg/kg	11.28±2.10#	65.4±7.9#	199.53±1.10#	186.21±0.81#
12	CCl_4 + Chloroform 50 mg/kg	6.55±1.39#	49.1±2.8#	175.91±0.27#	164.99±1.24#
13	CCl_4 + Chloroform 100 mg/kg	8.28±2.32#	55.7±2.1#	177.19±0.18#	168.06±1.20#
14	CCl_4 + Chloroform 200 mg/kg	9.11±1.27#	59.4±1.4#	180.01±0.22#	170.19±1.85#
15	CCl_4 + Silymarin- 100 mg/ kg	8.29±1.31#	56.1±2.7#	179.81±0.14#	171.80±1.21#

Values are expressed as Mean ± S.D (n = 6)
Toxicity (CCl_4) is compared with the normal;
**Values are statistically significant at P < 0.01 compared to control*

Table 2: Level of enzymatic antioxidants- *Ascorbate oxidase* & *Polyphenol oxidase*

S.NO	GROUP	*Ascorbate oxidase*	*Polyphenol oxidase*
1	Normal	27.23±0.57	2.19±0.199
2.	Ethanol 50 mg/kg	27.17±1.21	2.18±0.021
3	Ethanol 100 mg/kg	28.24±0.41	2.17±0.048
4	Ethanol 200 mg/kg	27.89±0.20	2.19±1.084
5	Chloroform 50 mg/kg	27.81±0.84	2.19±0.185
6	Chloroform 100 mg/kg	27.72±1.77	2.19±0.136
7	Chloroform 200 mg/kg	28.29±1.84	2.18±0.188
8	Carbon tetrachloride (CCl_4) 2 mg/kg	10.72±1.77*	17.97±2.842*
9	CCl_4 + Ethanol 50 mg/kg	14.01±1.21#	10.18±0.176#
10	CCl_4 + Ethanol 100 mg/kg	17.14±0.85#	9.10±1.128#
11	CCl_4 + Ethanol 200 mg/kg	19.71±0.84#	7.17±0.184#
12	CCl_4+ Chloroform 50 mg/kg	11.81±0.81#	14.19±1.107#
13	CCl_4+ Chloroform 100 mg/kg	12.01±0.27#	13.22±0.174#
14	CCl_4 +Chloroform 200 mg/kg	14.91±2.86#	11.08±0.292#
15	CCl_4 + Silymarin 100 mg/kg	14.71±0.82#	11.72±0.177#

Units: Peroxidase: µmoles/g sample; Ascorbate oxidase: unit/g sample, Polyphenol oxidase: µmoles/g tissue. Values are expressed as mean ± S.D (n = 6)

Table No. 3 Non-enzymatic antioxidant level- Total reduced glutathione and Vitamin C

S.NO	Groups	Total reduced glutathione	Vitamin C
1	Normal	62.28±0.72	254.23±1.21
2.	Ethanol 50 mg/kg	61.24±0.91	251.52±0.72
3	Ethanol 100 mg/kg	62.22±2.18	250.05±1.70
4	Ethanol 200 mg/kg	61.49±0.22	252.21±0.29
5	Chloroform 50 mg/kg	61.97±0.76	251.28±1.23
6	Chloroform 100 mg/kg	62.84±1.82	253.17±2.98
7	Chloroform 200 mg/kg	62.24±0.94	252.23±1.95
8	Carbon tetrachloride (CCl_4) 2 mg/kg	21.82±0.22*	105.68±1.82*
9	CCl_4 + Ethanol 50 mg/kg	45.71±1.84#	185.24±0.77#
10	CCl_4 + Ethanol 100 mg/kg	48.99±1.08#	192.55±2.72#
11	CCl_4 + Ethanol 200 mg/kg	55.76±0.49#	202.35±2.64#
12	CCl_4 + Chloroform 50 mg/kg	35.57±1.84#	165.55±0.34#
13	CCl_4 + Chloroform 100 mg/kg	39.06±0.92#	171.74±2.94#
14	CCl_4 + Chloroform 200 mg/kg	40.71±0.84#	181.23±0.27#
15	CCl_4 + Silymarin 100 mg/kg	41.70±1.61#	174.42±0.96#

Non- Enzymatic Antioxidants

The levels of non-enzymatic antioxidants of values are showed in Table-3. The total assessment of non-enzymatic antioxidants activity of *P. tomentosa* is noted to be effective. The level of total reduced glutathione and Vitamin C exhibited remarkable activity.

Units: Total reduced glutathione: µg/mg plant tissue, Vitamin C: µg/mg plant tissue. Values are expressed as Mean ± S.D (n = 6)

Toxicity (CCl_4) is compared with the normal; * Values are statistically significant at $P < 0.01$ compared to control. Treated groups are compared with toxicity (CCl_4) control; # Values are statistically significant at $P < 0.01$ compared to toxicity (CCl_4) control.

Carbon tetrachloride (CCl_4) is an effective hepatotoxin which causes centrilobular hepatic inflammation and necrosis (23). CCl_4 is assumed to cause oxidative stress by the generation of trichloromethyl (CCl_3*) free radicals in the body which results in oxidative damage of cell organelles and membrane, which initiates the chain reaction of lipid peroxidation. The changes associated with liver toxicity and damage (24).

Silymarin (100 mg / kg) was used as the standard hepatoprotective agent to confirm the reliability of the test system and also to compare the effectiveness of the test drug as, it has been used in the treatment of chronic or acute liver disease, as well as protecting the liver against toxicity (25).The hepatoprotective properties of Silymarin have been related to the scavenging of free radicals or inhibition of lipid peroxides formation (26).

In previous studies, *Perularia species* has been reported to be having many medicinal uses as

antibacterial, antifungal, and cytotoxic activity and hepatoprotective activity (27). Results of this study, shows that there were noticeable normalisation of the elevated antioxidant enzymes levels of SOD, CAT, GPx, GST, Ascorbate oxidase and Polyphenol oxidase and also non-enzymatic antioxidant such as total reduced glutathione and Vitamin C after oral administration of ethanol and chloroform leaf extract of *P. tomentosa* in CCl_4 intoxicated rats.

The major cause of oxidative damage is the production of reactive oxygen species (ROS) as a byproduct of ETC in the mitochondria which can be prevented by an antioxidant system: low molecular mass antioxidants (Ascorbic acid, Glutathione, and Tocopherols), and ROS-interacting enzymes such as *SOD*, peroxidases and catalases. The SOD enzyme destroys the superoxide radicals. Catalase catalyzes the dismutation of hydrogen peroxide in water and oxygen (28). *Glutathione peroxidase* acts as a radical scavenger, and membrane stabilizer. *Ascorbate oxidase* may participate in a redox system involving ascorbic acid and regulate the levels of oxidized and reduced glutathione and NADPH (29). *Polyphenol oxidase* catalyzes the aerobic oxidation of some phenolic compounds to quinones, and defense against water stress by scavenges H_2O_2 in chloroplasts (30). Excessive peroxidation causes increased glutathione consumption which is essential for recycling of antioxidants like vitamin E and vitamin C. Vitamin C is a water soluble antioxidant, scavenges peroxyradicals (31).

CONCLUSION

Based on all these finding it is suggested that the ethanol and chloroform extract of *P. tomentosa* leaves (test drug) has a potential source of natural antioxidant with high free radical scavenging potential. It may have great importance as therapeutic agents in preventing or slowing the oxidative stress related degenerative diseases and thus induce regeneration of liver cells. In future, *Pergularia tomentosa* may serve as a good pharmacotherapeutic agent.

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Conflict of interest
The authors declare that there is no conflict of interest.

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