Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(4): 3386-3393
Received: 04-05-2018
Accepted: 08-06-2018

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Anti-oxidant activity of ignored monocot grass *Kyllinga triceps* Rottb

Amit Upadhyay, Dr. Suman Jain, Neetu Bhalla and Kanika Arora

Abstract
In the present study the observed enhanced oxidative stress markers in the liver CCl_4 liver cirrhotic rats indicate enhanced oxidative stress in the liver of rats. *Kyllinga triceps* Rottb treatment for 60 days has decreased the intensity of oxidative stress markers in liver cirrhotic rats. Further to understand the protective role of *Kyllinga triceps* Rottb extracts (petroleum ether and etanolic), administration against CCl_4 induced oxidative stress in liver, the antioxidant status- antioxidant enzymes are analysed in the 7 experimental groups. Petroleum ether 200 mg/kg extract exhibits significant antioxidant activity while ethanolic extract 200 mg/ kg also exhibit good antioxidant potential but it is inferior than Petroleum ether 200 mg/kg extract.

Keywords: oxidative stress, ethanolic, *kyllinga triceps*, petroleum ether, ccl_4

Introduction
Herbal medicines are the use of plants and plant extracts as medicines. India is blessed with enormous varieties of medicinal and the aromatic plants, which may be attributed to the Indian climatic conditions. These plants are used as a potential source of many drugs in traditional Indian system of medicine.

The WHO is encouraging all the developing countries for using the traditional herbal medicines actively. There are 3000 plants used as medicine which are identified in the forests of India. The important chemical constituents from these plants are worth Rs 2000 crores in the US market. The plants are exhaustible source of medicine, but have remained unexplored. So, this provides a challenge for the medical and pharmaceutical sciences scientists for the search of new and potent drugs with minimal side effects. A lot of progress has been made in the phytochemical research from past few decades. Human body performs several endogenous defense mechanisms for protection against free radicals induced cell damage. The antioxidant enzymes-glutathione peroxidase, catalase, and superoxide dismutase metabolize oxidative toxic intermediates. Glutathione is an important water soluble antioxidant; it is synthesized from the amino acids glycine, glutamate and cysteine. Glutathione directly quenches reactive oxygen species as lipid peroxides, and also plays a significant role in xenobiotic metabolism. Plant is a major weed of improved pastures, but also occurs in crops, gardens, plantations and roadsides. It grows best in moist fertile soil that is seldom cultivated and in full sunshine. It is present in areas up to 7000 ft. elevation. The plant is naturalized primarily in gardens and lawns.

The rhizomes of plant *Kyllinga triceps* Rottb are fragrant, aromatic, sweet, astringent, bitter, refrigerant, febrifuge, antidiarrhoeal, diuretic, stomachic, anthelmintic, expectorant, demulcent and tonic. They are useful in vitiated conditions of pitta and vata, fever, cough, bronchitis, hepatopathy, splenopathy, diabetes, dermatitis, fistula and tumours.

Materials and Methods
Plant Materials
The species for the proposed study that is *Kyllinga triceps* Rottb were collected from Bhoora Khon area of Shivpuri District of Gwalior Division (M.P.) with the help of Mr. N.K. Pandey (R.O.) National Research institute for ayurvedic-siddha (CCRAS) Amkho, Gwalior. The species for the proposed study was identified as *Kyllinga triceps* by Dr. (Smt.) M.D. Gupta (Asst Director) and Mr. N.K. Pandey (R.O.) National Reseach Institute for Ayurveda and siddha (C.C.R.A.S.) under Ministry for Health and Family Welfare, Govt. of India, Amkho Gwalior (M.P.)

Preparation of plant extract

Preparation of the extract of *Kyllinga triceps* Rottb, powdered rhozme is done by using following solvents. Ethanolic extract cold percolation method was used for preparation of 80% alcoholic extract of dried *Kyllinga triceps* Rottb, rhozmes powder, rhizome powder were extracted with 80% ethanol for 24 hrs, which was filtered with 80 mesh nylon cloth. Rawmaterial and solvent ratio was 1:8, total extraction procedure was repeated for five times, clean and sterile conditions were maintained through out the extraction process so that their should be no chance of contamination. All the filterates obtained after extraction were combined and again subjected for filtration with 250 mesh nylon cloth, finally extract was obtained was concentrated with reduced pressure.

Petroleum Ether extract cold percolation method was used for preparation of petroleum ether extract of dried *Kyllinga triceps* Rottb, rhizomes powder, rhizome powder were extracted with Petroleum ether for 24 hrs, which was filtered with 80 mesh nylon cloth. Rawmaterial and solvent ratio was 1:8, total extraction procedure was repeated for five times, clean and sterile conditions were maintained throughout the extraction process so that their should be no chance of contamination. All the filtrates obtained after extraction were combined and again subjected for filtration with 250 mesh nylon cloth, finally extract was obtained was concentrated with reduced pressure.

Ethical aspects

The study was approved by the institutional ethical committee (protocol No. 891/Po/ac/05/CPCSEA).

Experimental animals

The rats were randomly divided into seven groups, comprising of six animals in each group.

Group-I. Rats of this group received normal saline (10ml/kg) for 60 days. This group served as a normal control.

Group-II. Rats of this group were intoxicated with carbon tetrachloride (CCl_4) with the dose of 0.3 ml/kg body weight/twice in a week, i.p. along with olive oil (50% v/v). This group served as a negative control.

Group-III. Rats of this group received Silymarin (25 mg/kg orally) daily once for 60 days with CCl_4 as given to group-II for 60 days. This group served as a positive control.

Group-IV. Rats of this group received petroleum ether extract of rhizome of *Kyllinga triceps* Rottb with the dose of 100 mg/kg orally daily once for 60 days with CCl_4 as given to group-II for 60 days.

Group-V. Rats of this group received petroleum ether extract of rhizome of *Kyllinga triceps* Rottb 200mg/kg orally daily once for 60 days with CCl_4 as given to group-II for 60 days.

Group-VI. Rats of this group received ethanol extract of rhizome of *Kyllinga triceps* Rottb 100mg/kg orally daily once for 60 days with CCl_4 as given to group-II for 60 days.

Group-VII. Rats of this group received ethanol extract of rhizome of *Kyllinga triceps* Rottb 200 mg/kg orally daily once for 60 days with CCl_4 as given to group-II for 60 days.

Assessment of antioxidant activity

Studies on antioxidant and oxidative stress

Preparation of tissue extracts for glutathione and lipid peroxidation

Immediately after separation of liver, 10% tissue homogenate was prepared in 0.15 M potassium chloride using homogenizer at 0 °C. The whole homogenate was used for estimation of glutathione and lipid peroxidation.

Lipid peroxidation

Principle: Lipid peroxidation was estimated as evidence by the formation of thiobarbutic acid reactive a substance like malondialdehyde (MDA) according method of Utely *et al.*

Reagents

1. 10% TCA
2. 0.67% TBA

Procedure: To 1.0 ml of the liver homogenate, 2.0 ml of TCA and 4.0 ml of TBA were added, heated in water bath for 30 minutes. After cooling and centrifugation, the absorbance of the supernatant was read at 535 nm. A reagent blank was prepared using water instead of tissue homogenate. The extent of lipid peroxidation was expressed as nmol MDA formed/mg protein, using a molar extinction co-efficient of MDA as 1.56 X 105 M^{-1} cm^{-1}.

Reduced glutathione

Principle: Total reduced glutathione content was measured following the method of Ellman's (1959). This method was based on the development of a yellow colour, when 5, 51-dithio-2-nitro benzoic acid (DTNB) reacts with the compounds containing sulphydryl groups with maximum absorbance at 412 nm.

Reagents

1. Phosphate buffer 0.2 M, pH: 8.0
2. 5% TCA
3. Ellman's reagent: 19.8 mg DTNB in 100 ml of 0.1% sodium citrate (prep. fresh)
4. GSH standard: 20 µg/ml

Procedure: 0.5 ml of liver tissue homogenate was deproteinized with 3.5 ml of 5% TCA and centrifuged. To 0.5 ml of the supernatant, 3.0 ml phosphate buffer and 0.5 ml of Ellman's reagent were added and the yellow colour developed was read at 412 nm. A series of standards (4-µ0 pg) were treated in a similar manner along with blank. Values expressed as µg GSH/mg protein.

4.4.2.4.1 Assay of antioxidants enzyme levels

Sample preparation

Ten percent of the liver tissue homogenate in 0.15 M potassium chloride was prepared at 0 °C and centrifuged in cold (0-4 °C) at 12,000 rpm for 45 min**,** in Remi **(**SL-**)** cooling centrifuge. The supernatant thus obtained into eppendorf tubes, labelled and stored at -20 °C and all the antioxidant enzymes were assayed at the earliest.

Assay of superoxide dismutase (SOD)

Principle: SOD activity was measured based on the ability of the enzyme to inhibit the autooxidation of pyrogallol. A modified procedure described by Marklund and Marklund and Marklund (1974) was adopted as followed by Soon and Tan (2002).

Reagents
1. 50 mM Phosphate buffer, pH:7.8 containing 1 mM EDTA
2. 10 mM Pyrogallol in 0.01 N HC1.

Procedure: The assay system contained 2.1 ml of buffer, 0.02 ml of enzyme source (35 pg protein) and 0.86 ml of distilled water. The reaction was initiated with 0.02 ml of pyrogallol and change in absorbance was monitored at 420 nm. The percent inhibition was calculated on the basis of comparison with a blank assay system. One unit of SOD was defined as that amount of enzyme required to inhibit the autooxidation of pyrogallol by 50% in standard assay system of 3 ml. The specific activity was expressed as units/min/mg protein.

Assay of glutathione peroxidase

Principle: A known amount of the enzyme preparation was allowed to react with H_2O_2 in the presence of GSH for a specified time period according to the method of Rotsrack (1973) and remaining GSH was measured by Ellman's method (1959) as described for GSH estimation.

Reagents
1. Phosphate buffer 0.4 M, pH: 7.0 containing 0.4 mM EDTA, 10 mM Sodium Azide
2. 2 mM GSH
3. 10% TCA
4. 0.2mM H_2O_2

Procedure: To 0.5 ml buffer, 0.2 ml enzyme source, 0.2 ml GSH, 0.1 ml H_2O_2 were added and incubated at room temperature for 10 min along with a control tube containing all reagents except enzyme source. The reaction was arrested by adding 0.5 ml of 10% TCA, centrifuged at 4000 rpm for 5 min. and GSH content in 0.5 ml of supernatant was estimated. The activity was expressed as µg of GSH consumed/min/mg protein.

Glutathione reductase

Principle: Glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) by NADPH to GSH. The activity of the enzyme was measured by following the oxidation of NADPH spectrophotometrically at 340 nm according to the method of Pinto and Bartley (1969).

Reagents
1. Potassium phosphate buffer, 0.25 M, pH:7.4
2. 25 mM EDTA
3. 50 mM GSSG
4. 1 mM NADPH

Procedure: The system contained 0.5 ml of buffer, 0.1 ml of EDTA, 0.1 ml of NADPH, 0.96 ml of distilled water and 0.1 ml of enzyme source (150 µg protein). The reaction was initiated by the addition of 0.24 ml GSSG. The change in absorbance was recorded at 1 min intervals at 340 nm for 5 min. The specific activity is expressed as µmol of NADPH oxidized/min/mg protein using an extinction coefficient for NADPH of 6.22 cm^{-1} $mmol^{-1}$.

Glutathione-S-transferase

Principle: Glutathiione-S-Transferase activity was measured by monitoring the increase in the absorbance at 340 nm using l-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig *et.al*. (1974)

Reagents
1. 0.14 M Sodium phosphate buffer, pH:6.5
2. 0.01 M CDNB dissolved in 50% ethanol
3. 30 mM GSH

Procedure: The assay system contained 1.7 ml of buffer, 0.2 ml GSH and 0.04 ml enzyme source (40 µg protein). The reaction was initiated by 0.06 ml CDNB. The change in absorbance was recorded at 1 min intervals at 340 nm for 5 min and the activity was calculated using an extinction coefficient of CDNB-GSH conjugate as 9.6 mM^{-1} and expressed as mmoles of CDNB-GSH conjugate formed/min/mg protein.

Assay of catalase

Principle: Catalase catalyses the breakdown of H_2O_2, and it was measured spectrophotometrically at 240 nm following the method of Beers and Seizer (1952).

Reagents
1. 0.05 M Sodium phosphate buffer, pH:7.0
2. 0.059 H202 in buffer.

Procedure: The assay system contained 1.9 ml buffer and 1.0 ml H_2O_2. The reaction was initiated by addition of 0.1 ml enzyme source (45 µg protein). The decrease in absorbance was monitored at 1 min interval for 5 min at 240 nm and activity was expressed as "n" moles of H_2O_2 decomposed/min/mg protein.

Result and Discussion

Liver antioxidant enzymes

The data presented in Table:1 (Fig. 1 to 5) showed analysis of various antioxidant enzymes like Catalase (CAT), Superoxide dismutase (SOD), Gluthione peroxidise (GPx), Glutathione reductase (GR), Glutathione-S- transferase (GST) activities were studied in liver tissue in all groups. Group II (CCl_4 treated alone) showed significant ($p<0.05$) decrease in the activity of CAT, SOD, GPx, GR and GST with Normal control group, whereas, group IV, V, VI and VII showed significant ($p<0.05$) increase in the antioxidant activity over the group II. Group VII (CCl_4 + PE-KTR 200 mg/kg) showed a significant increase in the activity of CAT, SOD, GPx, GR and GST over group IV (CCl_4 + AE-KTR 100 mg/kg), group V (CCl_4 + AE-KTR 200 mg/kg) and group VI (CCl_4 + AE-KTR 100 mg/kg) and not significantly different with group III (CCl_4 + silymarin treated group). Antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 -induced hepatopathy. The body has an effective defence mechanism to prevent and neutralize free radicals-induced damage. This is accomplished by a set of endogeneous antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase. These enzymes constitute a mutually supportive team of defense against reactive oxygen species (ROS). SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of $O2^{-\cdot}$. GPx is a seleno enzyme two third of which (in liver) is present in the cytosol and one third in the mitochondria. It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. GST plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. GR is concerned with the

maintenance of cellular level of GSH (especially in the reduced state) by effecting fast reduction of oxidized glutathione to reduced form. In CCl_4 induced hepatotoxicity, the balance between ROS production and antioxidant defences may be lost hence oxidative stress results which, through a series of events deregulate cellular functions leading to hepatic necrosis. It is generally thought that CCl_4 toxicity is due to reactive free radical ($CCl_3^{\cdot}$), which is generated by its reductive metabolism by hepatic cytochrome P450. The reactive intermediate is believed to cause lipid peroxidation and breakdown of cellular membranes. In the present study treatments of Kyllinga triceps Rottb extracts for 60 days not showed variations significantly over the untreated control with reference to the liver antioxidant enzyme levels. $CC1_4$ treatment showed significant (Table. 1 and Fig. 1 to 5) depletion in the antioxidant enzymes like SOD, CAT, GPx, GR, and GST levels in the liver tissue. $CC1_4$ along with Kyllinga triceps Rottb extracts treatment showed significant ($p<0.05$) recovery in the liver antioxidant enzyme levels compared to $CC1_4$ treated ones. These trends confirm that $CC1_4$ depleted liver antioxidant enzyme levels were normalised by Kyllinga triceps Rottb extracts treatment. The depletion of antioxidants observed in different experimental models was significantly restored with KTR extract treatment. Therefore, they have reason to believe that the antioxidant activity of Kyllinga triceps Rottb might be directly or indirectly associated with maintenance or preservation of membrane integrity, which might help to prevent the elevation of serum marker enzymes observed during inflammation. In respect to antioxidant enzyme levels in KTR extract treatment, our results showed that they are almost nearer to standard drug treated group (Siylamarin). To our knowledge till date no reports were available showing protection by Kyllinga triceps Rottb against $CC1_4$ toxicity. So more studies are required to conform the antioxidant activity of Kyllinga triceps Rottb.

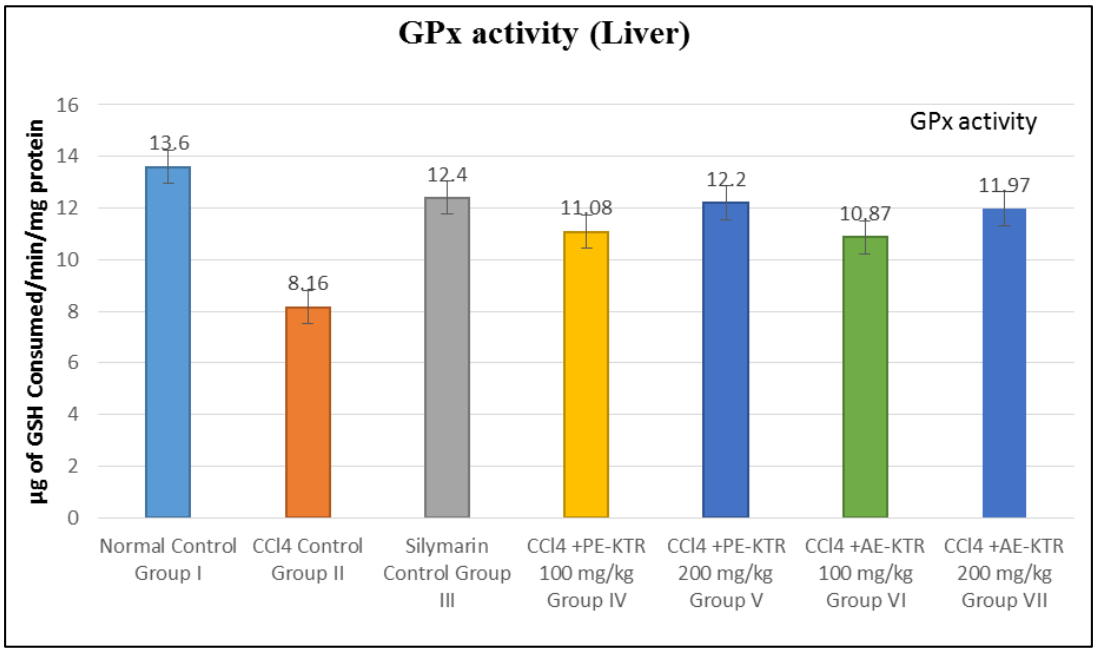
Table 1: Effect of Kyllinga triceps Rottb. Extract on antioxidant enzyme activities in the liver of rats treated with CCl_4.

Name of Parameter	Group I (Normal Control)	Group II (CCl_4 control)	Group III (CCl_4 +Silymarin control)	Group VI (CCl_4 + PE-KTR 100 mg/kg)	Group VII (CCl_4 + PE-KTR 200 mg/kg)	Group IV (CCl_4 + AE-KTR 100 mg/kg)	Group V (CCl_4 + AE-KTR 200 mg/kg)
GPx (µg of GSH/min/mg protein)							
Mean	13.6^a	8.16^d	12.4^b	11.08^c	12.2^b	10.87^c	11.97^b
S.D.	±0.59	±0.26	±0.5	±0.59	±0.67	±0.56	±0.64
GST(µM/min/mg protein)							
Mean	6.65^a	2.65^d	5.86^b	5.01^c	5.74^b	4.15^c	5.04^b
S.D.	±0.14	±0.2	±0.05	±0.19	±0.24	±0.17	±0.09
GR (µM/min/mg protein)							
Mean	0.145^a	0.0641^d	0.136^b	0.119^c	0.134^b	0.111^c	0.124^b
S.D.	±0.001	±0.001	±0.0037	±0.0032	±0.009	±0.007	±0.0052
CAT (µM H_2O_2/min/mg protein)							
Mean	68.7^a	28.236^e	65.48^b	65.12^d	65.89^c	60.65^d	64.56^c
S.D.	±0.372	±0.257	±0.148	±0.365	±0.554	±0.654	±0.248
SOD (U/mg protein)							
Mean	13.25^a	5.12^e	11.97^b	10.52^d	11.91^c	9.57^d	10.11^c
S.D.	±0.274	±0.1894	±0.2	±0.456	±0.561	±0.241	±0.358

Values are mean ± S.E. M

Values with different superscripts within the column are significantly different at $P<0.05$ (Duncan's Multiple Range Test)

GPx-Glutathione peroxidise, GST- Glutathione S –transferase, GR- Glutathione reductase, CAT- Catalase, SOD- superoxide dismutase



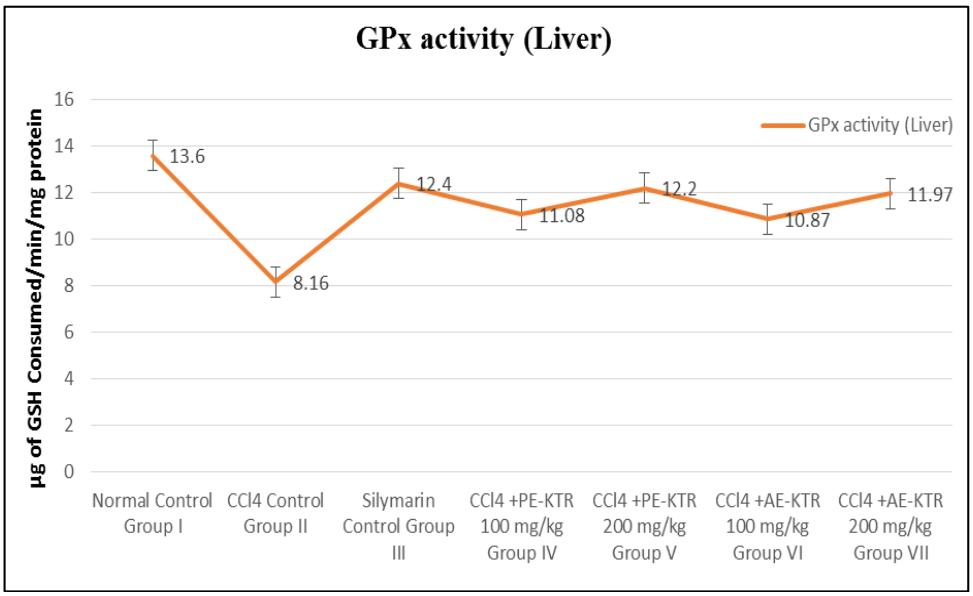
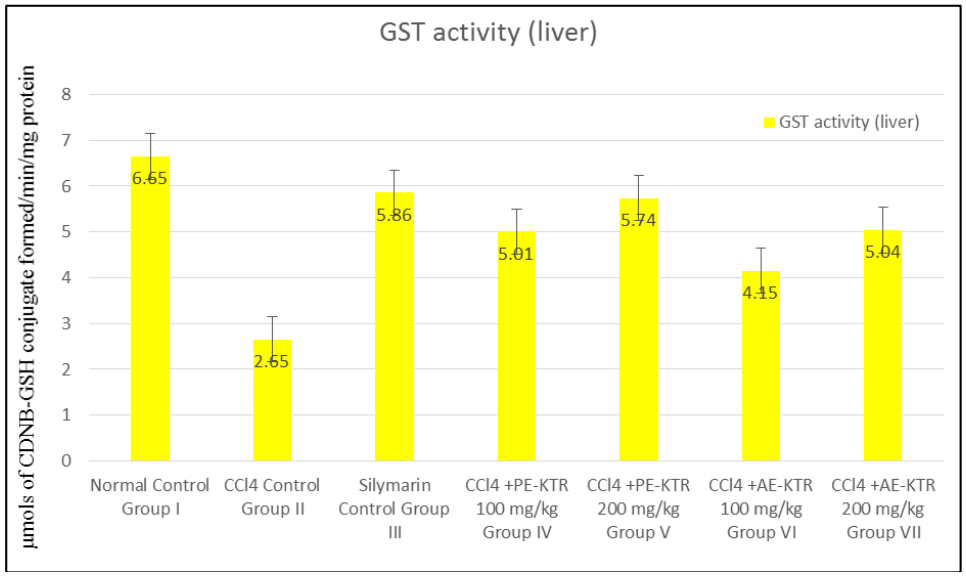


Fig 1: Effect of KTR extracts on liver GPx activity of rats treated with CCl_4 Group I - Normal Control, Group II - CCl_4 control, Group III - CCl_4 +Silymarin control, Group IV - CCl_4 + PE-KTR 100 mg/kg, Group V - CCl_4 + PE-KTR 200 mg/kg, Group VI - CCl_4 + AE-KTR 100 mg/kg, Group VII - CCl_4 + AE-KTR 200 mg/kg



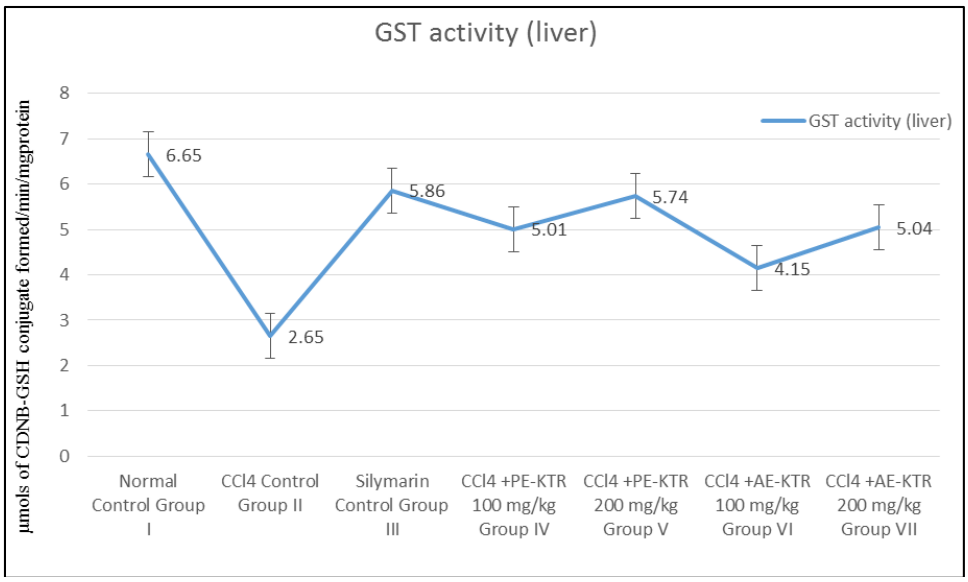


Fig 2: Effect of KTR extracts on liver GST activity of rats treated with CCl_4Group I - Normal Control, Group II - CCl_4 control, Group III - CCl_4 +Silymarin control, Group IV - CCl_4 + PE-KTR 100 mg/kg, Group V - CCl_4 + PE-KTR 200 mg/kg, Group VI - CCl_4 + AE-KTR 100 mg/kg, Group VII - CCl_4 + AE-KTR 200 mg/kg.

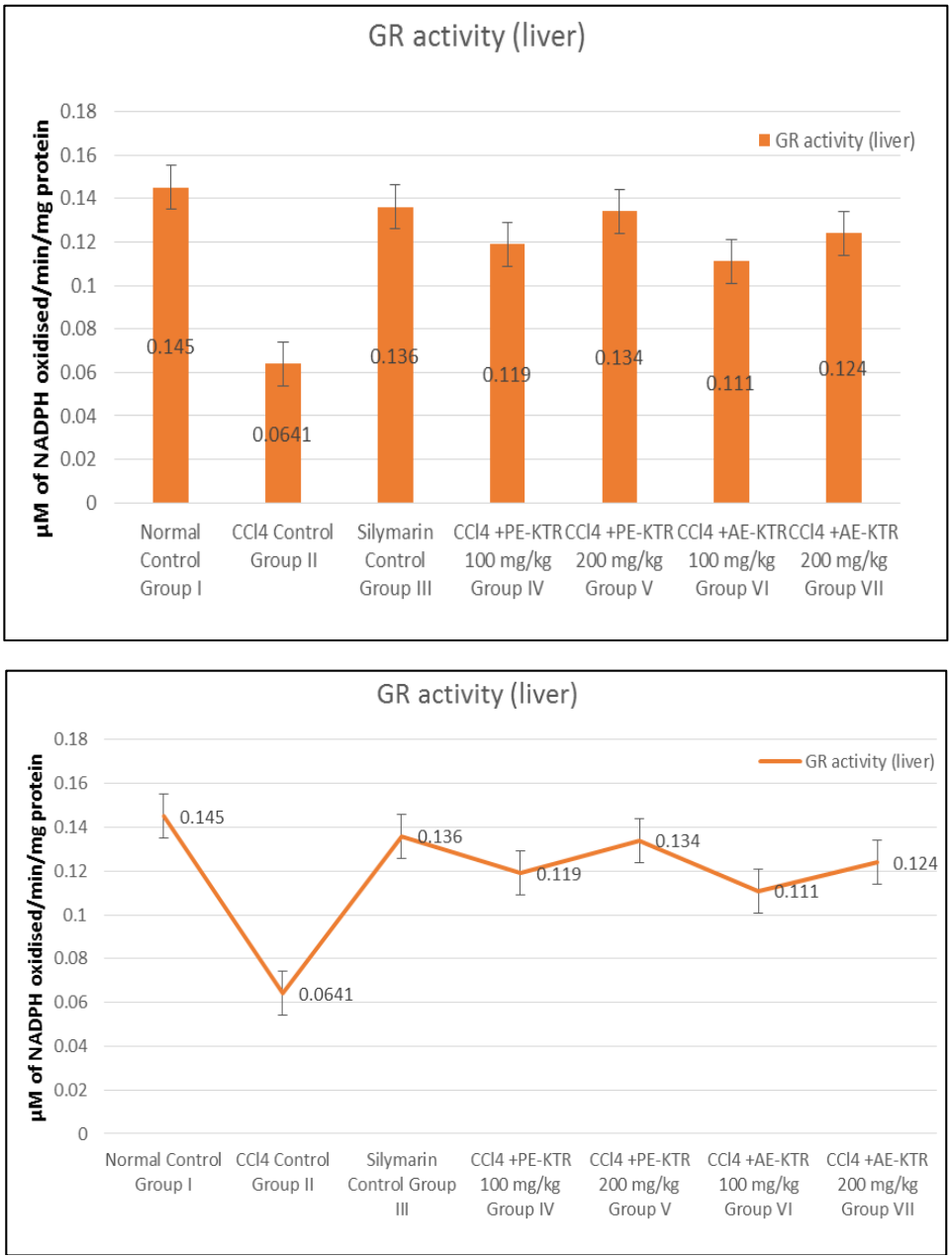
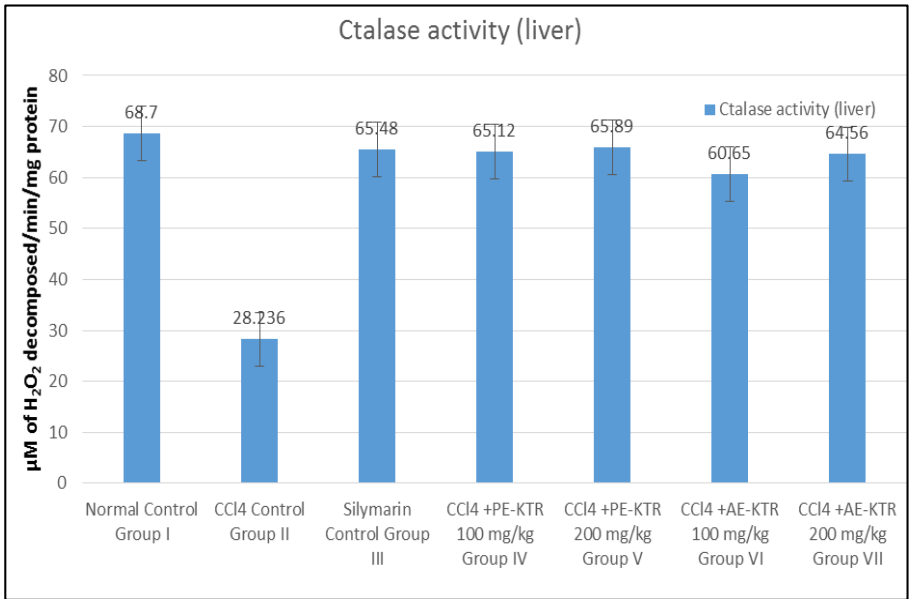


Fig 3: Effect of KTR extracts on liver GR activity of rats treated with CCl_4Group I - Normal Control, Group II - CCl_4 control, Group III - CCl_4 +Silymarin control, Group IV - CCl_4 + PE-KTR 100 mg/kg, Group V - CCl_4 + PE-KTR 200 mg/kg, Group VI - CCl_4 + AE-KTR 100 mg/kg, Group VII - CCl_4 + AE-KTR 200 mg/kg.



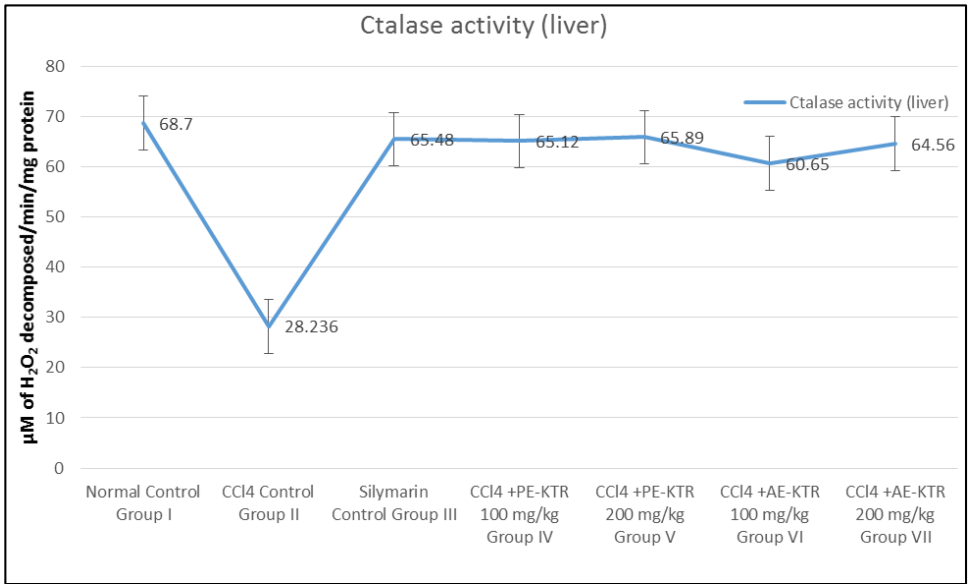
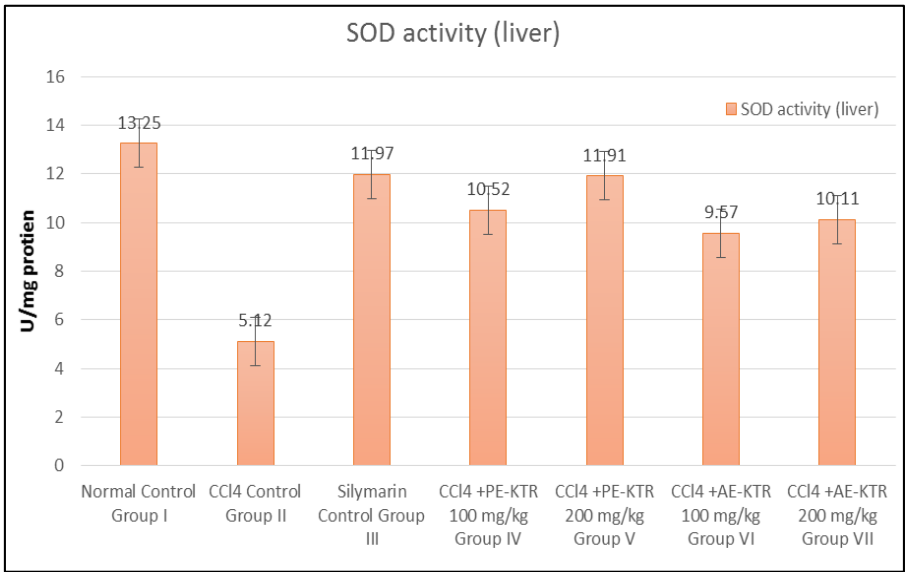


Fig 4: Effect of KTR extracts on liver catalase activity of rats treated with CCl_4Group I - Normal Control, Group II - CCl_4 control, Group III - CCl_4 +Silymarin control, Group IV - CCl_4 + PE-KTR 100 mg/kg, Group V - CCl_4 + PE-KTR 200 mg/kg, Group VI - CCl_4 + AE-KTR 100 mg/kg, Group VII - CCl_4 + AE-KTR 200 mg/kg.



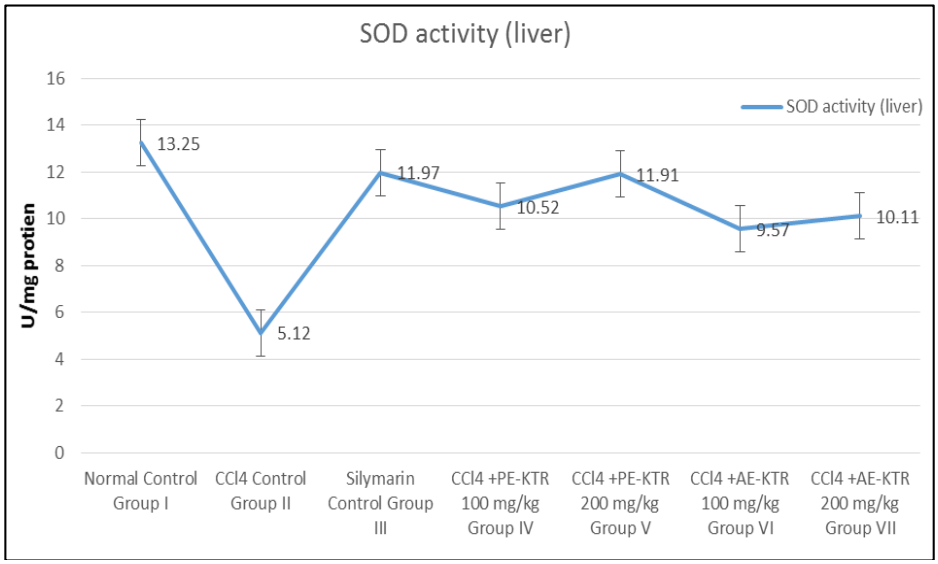


Fig 5: Effect of KTR extracts on liver SOD activity of rats treated with CCl_4Group I - Normal Control, Group II - CCl_4 control, Group III - CCl_4 +Silymarin control, Group IV - CCl_4 + PE-KTR 100 mg/kg, Group V - CCl_4 + PE-KTR 200mg/kg, Group VI - CCl_4 + AE-KTR 100 mg/kg, Group VII - CCl_4 + AE-KTR 200 mg/kg

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