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The Hepatoprotective Effect of The Polyphenolic Compounds in the Roots of *Trichilia connaroides* Wight and Arn

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Abstract:

Trichilia species are used in the treatment of liver disorders and as a tonic in the traditional medicine. The present study was designed to evaluate the hepatoprotective effects of aqueous extract of *T. connaroides* roots on carbon tetrachloride induced hepatotoxicity in comparision with the known hepatoprotective agent Liv-52. Carbon tetrachloride induced changes in serum enzymatic levels of aspartate amino transferase , alanine amino transferase , alkaline phosphatase and total protein were restored towards normal levels by the extract. The biochemical observations were supported by the histopathological examination of rat liver sections. The results indicate that the extract offers hepatoprotection in a dose-dependent manner. The effect was comparable to that produced by Liv-52. Simultaneously, *in-vitro* anti-oxidant activity of the extract as evaluated in terms of reducing power, radical scavenging activity and chelating activity on Fe^{+2} also support its hepatoprotective action. Phytochemical examination of the extract revealed the presence of antioxidant phenolics.

Keywords: *Trichilia connaroides,* Carbon tetrachloride, Hepatoprotective activity, Antioxidant activity, Histopathology.

1. Introduction

Liver is a key organ of metabolism and detoxification. Continuous exposure to a variety of environmental toxic agents enhances hepatic injury. A growing interest has emerged around the globe in rediscovering medicinal plants as useful therapeutic agents for the prevention of such injury[1]. Eventhough modern medicine is advancing at a fast pace no effective drugs are available, to stimulate liver functions and to offer protection to the liver from the damage or help to regenerate hepatic cells[2]. Therefore, many folk remedies of plant origin are tested for their potential anti-oxidant and hepatoprotective liver damage in experimental animal model [1]. A large number of medicinal preparations are recommended for the treatment of liver disorders due to the lack of reliable liver protective drugs [3].The Meliaceae plant family has long been used in India for its medicinal properties, contains 40 genera and 600 species [4,5] distributed in sub-Himalayan tract from Kumaun eastward, Sikkim up to 4000 ft, Khasia Hills, Manipur,E. Ghats in the forests of Godawari and Vizagapatnam up to 4,500 ft. W.Ghats from Poona Southwards through the Nilgiris and Anamalais to Tranvancore, up to 6,000 ft. It is also distributed in Burma, Tonkin, Cambodia, Malay Peninsula and Sumatra[6]. *T. connaroides* Wight and Arn. is the only species in the genus to occur in India[6].

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Roots of *T. connaroides* are used as a Chinese drug to treat arthritis, pharingitis, tonsillitis and other ailments[7]. They are also used as tonic in traditional Indian medicine[5]. A decoction of the leaves is taken in cholera [5]**.** Roots of *Trichilia. emetica* vahl. syn. *T. roka* Chiov are used for the treatment of liver disorders in the folk medicine of Mali. So its aqueous root decoction has been studied for the hepatoprotective effects and found to be quite active. The activity was attributed to the presence of the polyphenols [8, 9]. Since *T. connaroides* belongs to the same genus and contains polyphenolic acids hence is was considered as the subject of this study.
To date possible anti-oxidant and hepatoprotective activity of *T. connaroides* has not reported. The present communication investigates the hepatoprotective and *in vitro* anti-oxidant activities of the aqueous extract of *T. connaroides* as well as the effects of the phenolic acid components of its root.

2.Materials and Methods

2.1 Plant material and preparation of plant extract

Roots of *T. connaroides* were collected from Kumaun region, India and identified at Forest Research Institute (FRI) Dehradun-Uttaranchal vide herbarium no. M-29. Shade dried and powdered roots (400 g) were extracted with boiling water for three times.
The obtained extract (ARE) was then filtered and dried to powder in a freez drier (Labconco Corp.Kansas City, Mo.U.S.A). The dried extract (5g, % yield=1.25) was dissolved in distilled water in the required amount at the time of dosing.

2.2 Chemicals
Carbon tetrachloride (CTC), HCl, NaOH, Butylated Hydroxy Toluene (BHT), EDTA, Citric acid, Diethyl ether, Methanol were procured from E-Merk (India) Limited Mumbai. 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical and phenolic acids standard were procured from the Sigma Aldrich USA.
2.3 Experimental Animals
Male albino rats (140-230 g) of Sprague -Dawley strain were procured from the Laboratory Animal Unit, Govind Ballabh.Pant University of Agriculture & Technology., Pantnagar, Uttarakhand and kept in laboratory for one week to acclimatize in the new environment. During this period they were fed with standard rat diet (Lipton, India) and water *ad libitum*. Lighting was regulated to provide equal hours of light and dark. The study protocol was approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (330/CPCSEA). All the experiments were performed in morning according to current guidelines for the care of the investigation of experimental pain in conscious animals [10].

3. Hepatoprotective Activity

Treatment Schedule
Carbon tetrachloride (CTC) induced acute toxicity: CTC was diluted with liquid paraffin (1:1) before administration. The animals were divided into six groups of six each. The animals were then subjected to either one of the following treatments for seven days:
Group 1: Distilled water (10ml/Kg body weight)
Group 2: Distilled water (10 mL/Kg b. wt + CTC (1 mL/Kg b. wt, i.p.)
Group 3: ARE [Aqueous root extract of *T. connaroides*) (100 mg/Kg b. wt) + CTC (1 mL/Kg b. wt, i.p.)
Group 4: ARE (200 mg/Kg b. wt) + CTC (1 mL/Kg b. wt, i.p.)
Group 5: ARE (400 mg/Kg b. wt) + CTC (1 mL/Kg b. wt, i.p.)
Group 6: Liv-52[Himalaya Drug Company, India] (2.5 mL/Kg b. wt) as a standard drug + CTC (1 mL/Kg b. wt, i.p.)
The drugs were administered every day in the morning between 8-9. A.M. Weights of all the rats were taken on the first and the final day before feeding. On the 8th day morning, all the rats were anaesthetized with pentobarbital sodium (40 mg/kg b.wt. i.p.) and blood was collected from the orbital sinus through vein puncture.

Livers of the sacrificed animals were removed and preserved in 10% formalin solution for histopathological study. General well being and behavior of the animals were observed daily throughout the period.

3.1 Assessment of liver function

The hepatoprotective effect of the extract was evaluated by the assay of liver function biochemical parameters (total protein, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and alkaline phosphatase (ALP) activities using standard UV-auto-test kits (Span Diagonistic, India) and histopathological studies of the liver.

4. Antioxidant activity

The antioxidant activity of ARE(aqueous root extract) was evaluated in terms of following three methods.

4.1 **Reducing Power**

The reducing power of ARE was determined using the earlier reported method [11]. 0.5 ml of different concentrations of ARE and standard BHT (5,10,15,20 and 25mg/ml) were mixed separately with 2.5 mL of phosphate buffer (200mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50°C and 2.5 ml of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation 650 x g for 10 min. The upper layer (5mL) was mixed with 5 mL of distilled water and 1mL of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700 nm using the UV-Visible spectrophotometer.

4.2 **Radical Scavenging Activity**

The scavenging effect on DPPH radical was determined according to the methods developed earlier [11]**.** Various amounts of ARE and standard BHT (5, 10, 15, 20 and 25 mg) were mixed with 5 ml of 0.004% methanol solution of DPPH. Each mixture was incubated for 30min in the dark and the absorbance of the sample was read at 515 nm using the UV-visible spectrophotometer. The DPPH solution was freshly prepared and kept in the dark at 4°C between the measurements.

DPPH scavenging activity (%) is calculated as $[1 - (A_t / A_o)] \times 100$

(Where A_t is the absorbance of the sample at 515 nm, and A_o is the absorbance of the control at 515 nm).

4.3 **Chelating activity**

The chelating activity of the ARE on ferrous ions Fe^{+2} was measured according to the method of Decker and Welch [12] .Aliquots of 1ml of different concentrations (5, 10, 15, 20 and 25 mg/ml) of the ARE were mixed with 3.7 mL of deionized water. The mixture was left for reaction with $FeCl_2$ (2 mM, 0.1 ml) and ferrozine (5mM, 0.2 ml) for 10 min at room temperature, and then the absorbance was measured at 562nm. The chelating effect was compared with that of EDTA at a level of 0.01 mM and citric acid at a level of 0.025 M. Chelating activity (%) is calculated as $[1 - (A_t / A_o)] \times 100$

(Where A_t is the absorbance of the sample at 562 nm and A_o is the absorbance of the control at 562 nm).

5. Phytochemical Analysis

The dried and coarse powdered roots of *T. connaroides* were refluxed with 2N HCl to liberate the free phenolics for 2hrs at 70-75°C, cooled to room temperature, centrifuged and then filtered. Filtrate was neutralized with 2N NaOH solution and extracted with diethyl ether. The organic layer was then dried and suspended in water for HPLC analysis. Benzoic, chlorogenic, gallic, ferulic, *o*-coumaric, *p*-coumaric, p-hydroxy benzoic, protocatechuic, syringic and vanillic acids were used as phenolic acid standards.[13]

6. Statistical Analysis

Values are given as mean ± S.D. Statistical analysis was done by Student's t-test in Windows Excel 2003.

7. Results

7.1 Hepatoprotective Activity (Biochemical examination)

Effect of ARE on CTC-induced hepatotoxicity was evaluated in rats and changes in serum biochemical parameters are presented in **Table 1.** CTC significantly increased the levels of serum enzymes viz. ALT, AST, ALP and significantly reduced the level of total serum protein in group II rats as compared to group I (normal) indicating the sign of hepatotoxicity. ARE-treatment considerably reduces the level of enzymes in the groups III, IV and V, but statistical significance was not reached but level of serum protein was significantly increased in group IV and V. The results were less significant when compared with the Liv-52.

7.2 Histopathological examination

Photomicrographs of haematoxylin-eosin stained liver tissue show the normal liver with normal hepatocytes arranged in hepatic chords **(Fig 1-A)** Liver sections from CTC treated control revealed massive degeneration and necrotic changes. The degenerative changes consisted of small to large vacuoles in the hepatocytes, nucleus was pushed to one side of the hepatocytes. Mononuclear cellular (lymphocytes) infiltration, deposition of collagen fibers and mild hyperemia was also observed **(Fig 1-B).** Normal architecture of the liver is restored by higher doses of ARE in group IV and V. Microscopic lesions in the liver of the ARE treated group at a dose rate of 100mg/kg b.wt were almost similar as in the CTC treated group at 8th day post experimentation **(Fig 1-C).** Microscopic examination of group IV liver section exhibited less intense necrotic changes. Vacuolar degeneration and perivascular inflitration of mononuclear cells was also reduced **(Fig 1-D),** whereas liver section of Group V rats marked reduction in vacuolar degeneration and necrotic changes and, normal parenchyma is observed as compared to the above mentioned groups **(Fig1-E).** Reduced deposition of collagen fibers was comparable with Liv-52 treated group **(Fig 1-F).**

7.3 Effect on liver and body weights in rats

The liver weight of CTC treated rats (Group II) increased significantly as compared to normal (Group I) showing the sign of hepatic damage. The liver weight of Liv-52 treated group decreased significantly as compared to CTC treated group showing the protective effect of Liv-52. But the body weight of all the rats remains unaltered (**Table 2**).

7.4 Antioxidant activity

7.4.1 Reducing Power:

ARE exhibited moderate to good reducing power compared to BHT in a dose-dependent manner **(Table- 3 and Fig- 2)**. The reducing power of ARE might be result of their hydrogen- donating ability of its components [11], which reduces the Fe^{+3} /ferricyanide complex to the ferrous form (Fe^{+2}). The Fe^{+2} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm [14].

7.4.2 Radical Scavenging Activity

ARE exhibited moderate radical scavenging activities at lower concentration, but at highest concentration its radical scavenging is comparable with the BHT (**Table 4 and Fig-3**).

7.5. Chelating activity

ARE showed chelating activity on Fe^{+2} in dose dependent manner as illustrated in **Table 5, Fig 4.** The chelating activity of ARE at all concentration was higher than that of EDTA at 0.01mM and citric acid at 0.025mM (35.01 5% 30.79 % respectively).

7.6. Phytochemical Examination of the T. connaroides extract

Phytochemical analysis of ARE reveals the presence of phenolic acids- chlorogenic, ferulic, gallic, *p*-coumaric, protocatechuic and p-hydroxy benzoic acids.

8. Discussion

In the present work, aqueous extract of *T. connaroides* (ARE) was evaluated for hepatoprotective activity against CTC induced liver damage. Hepatotoxic effects of CTC are mainly due to its active metabolite, trichloromethyl radical [15,16] which binds covalently to the macromolecules and induces peroxidative degradation of the membrane lipid of the peroxide and the products like malodialdehyde are formed. This lipid peroxidative damage of the biomembranes is one of the major causes of CTC-induced hepatotoxity [17, 18]. AST and ALT are the important indicators of liver damage as they are released into the blood circulation following injury to the liver [19].

It had been shown that ARE exerted its action by preserving the structural integrity of the hepatocellular membrane resulting in the reduction of enzymatic level in the blood compared to CTC- treated rats. Significant increase in the level of total serum protein in ARE treated groups is also considered as a sign of hepatoprotection. This stimulation of protein synthesis can be depicted as hepatoprotective mechanism, which accelerates the regeneration process and production of the liver cells[20]**.** CTC induced a significant increase in liver weight, which is due to the blocking of secretion of hepatic triglycerides into the plasma [21]. ARE at higher doses prevented the increase in the liver weight of pretreated with CTC. Considering all the results, we can confirm that ARE exerted a clear protective action against CTC- induced hepatic damage.

Antioxidants are known to interrupt free-radical chain oxidation and to donate hydrogen from phenolic hydroxyl groups, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids [22]. The antioxidant activity is evaluated in terms of reducing power, DPPH radical scavenging potential and chelating activity. DPPH has been widely used to evaluate the free radical scavenging capacity of antioxidants [23, 24]. The ferrous state of iron accelerates lipid oxidation to reactive free radicals. Fe^{+2} ion also produces radicals from peroxides [25] and is the most powerful pro-oxidant among various species of metal ions [26]. Ferrozine, a chelating agent, was used to indicate the presence of chelator in the reaction system. Ferrozine forms a complex with free Fe^{+2} but not with Fe^{+2} bound to extracts. In the presence of chelating agents, the complex formation of ferrous and ferrozine is disrupted, resulting in a decrease in red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator[27].

The antioxidant and hepatoprotective activities were studied to correlate each other. The lipid peroxidation is accelerated when free radicals are formed as the result of losing a hydrogen atom from the double bond of the unsaturated fatty acids. Scavenging of free radicals is one of the major anti-oxidant mechanisms to inhibit the chain reaction of lipid peroxidation. The free radical scavenging activity of ARE was evaluated by DPPH assay. DPPH is a well known abstractor of hydrogen. DPPH scavenging activity suggested that ARE contains the free radical scavengers which counter the pathological changes caused by the generated free CCl_3 radicals. Scavenging of DPPH radical is related to the inhibition of lipid peroxidation[28]. Antioxidants are known to interrupt the free-radical chain oxidation and to donate hydrogen from phenolic hydroxyl groups, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids [22]. The reducing power of ARE might be due to the presence of compounds having hydrogen- donating ability [11]**.** Antioxidants present in the ARE reduced the Fe^{+3} /ferricyanide complex to the ferrous form (Fe^{+2}) [14]. Reducing power and chelating activity supplemented the radical scavenging effect of ARE.

Phenolic groups play an important role in anti-oxidant activity [11] .These polyphenolic compounds in the cell can function as antioxidants and anti-prooxidants by scavenging reactive oxygen species via enzymatic and non-enzymatic reactions [29]. It has been reported earlier, that antioxidants are responsible for hepatoprotective action[30]**.** Thus our findings suggest that the free radical scavenging and anti-oxidant activities could be the possible mechanism for the hepatoprotective activity of ARE which may be attributed to the presence of phenolic compounds.

9. Conclusion

Trichilia connaroides showed hepatoprotective action supported by biochemical parameters, histopathology along with the antioxidant potential. The hepatoprotective effect was found to be comparable to Liv-52 administered as a standard drug. Further studies are in progress for understanding of the mechanism of action.

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Table 1
Effect of aqueous extract of *T. connaroides* root (ARE) on carbon tetrachloride (CTC, 0.5 ml/kg b.wt.) induced serum biochemical changes in rats. (mean±S.E, n=6)

Group	Dose	ALP (KA)	ALT(IU/L)	AST (IU/L)	Total serum protein (g/dl)
I	Water (10 ml/kg)	8.58 ± 0.54	45.6±5.57	165.83±8.9	8.64 ± 0.414
II	CTC (0.5 ml/kg) + water (10 ml/kg)	24.58 ± 2.5^{c}	329.58±3.8^{c}	294.2±22.9^{c}	3.39 ± 0.12^{c}
III	ARE (100 mg/kg) + CTC (0.5 ml/kg)	22.45 ± 2.7	320.25±15.6	253.5±20.9	3.87 ± 0.29
IV	ARE (200 mg/kg) + CTC (0.5 ml/kg)	20.2 ± 1.96	294.0 ±18.73	226.6±20.2	4.66 ± 0.39^{x}
V	ARE (400 mg/kg) + CTC (0.5 ml/kg)	18.0 ± 3.98	260.25±33.1	241.2±31.5	5.72 ± 0.43^{y}
VI	Liv-52 (2.5 ml/kg) + CTC (0.5 ml/kg)	15.2 ± 4.41	250.0±16.68^{y}	216.55± 26.7^{y}	7.40 ± 0.46^{z}

ALP – Alkaline phosphatase; ALT–Alanine amino transaminase; AST– Aspartate amino transaminase; ARE –Aqueous root extract; CTC-Carbon tetra chloride. Student's t-test – $P^{a}<0.05$, $P^{b}<0.01$, $P^{c}<0.001$ vs group I and $P^{X}<0.05$, $P^{y}<0.01$, $P^{z}<0.001$ vs group II

Table 2
Effect of aqueous extract of *T. connaroides* root (ARE) on carbon tetrachloride (CTC, 0.5 ml/kg b.wt.) induced liver and body weights in rats.(mean ± S.E, (n=6).

Group	Dose	Wet liver weight (g)	Body weight(g) 1st day	Body weight(g) 8th day
I	Water (10 ml/kg)	5.78± 0.33	210.83 ± 6.50	203.3 ± 8.75
II	CTC (0.5 ml/kg) + water (10 ml/kg)	7.02 ± 0.58^{a}	188.33 ± 10.38	181.0 ± 0.64
III	ARE (100 mg/kg) + CTC (0.5 ml/kg)	6.37 ± 0.53	200.0 ± 6.454	167.5 ± 5.62
IV	ARE (200 mg/kg) + CTC (0.5 ml/kg)	5.98 ± 1.01	200.8 ± 6.50	176.6 ± 6.29
V	ARE (400 mg/kg) + CTC (0.5 ml/kg)	6.08 ± 0.197	197.5±11.67	168.75 ± 8.8
VI	Liv-52 (2.5 ml/kg) + CTC (0.5 ml/kg)	5.73 ± 0.34^{x}	171.66±11.66	173.0 ± 9.02

Student's t-test- $P^{a}<0.05$ vs. group I and P^{x} vs group II.

Table 3
Reducing power of aqueous extract of roots of *T. connaroides* (ARE) and butylated hydroxy toluene (BHT) at different concentrations.

Weight of ARE (mg/ml)	Reducing power	
	ARE	BHT
5	0.833 ± 0.00	0.846 ± 0.30
10	0.92 ± 0.00	1.00 ± 0.00
15	1.00 ± 0.02	1.24 ± 0.01
20	1.25 ± 0.00	1.48 ±0.01
25	1.47 ± 0.00	1.79 ± 0.05

Table 4
Radical scavenging ability of roots of *T. connaroides* (ARE) and butylated hydroxyl toluene (BHT) at different concentrations.

Weight of ARE (mg)	Radical scavenging activity (%)	
	ARE	BHT
5	58.3 ± 0.344	91.15±0.38
10	70.24±1.36	91.86±0.07
15	74.91±1.198	92.25±0.08
20	81.62±0.55	92.7±0.09
25	86.27±1.04	93.23±0.14

Table 5 Chelating activity on Fe^{+2} of aqueous extract of T. *connaroides* (ARE) at different concentrations.

Weight of ARE (mg/ml)	Chelating activity on Fe^{+2} of ARE (%)
5	58.4 ± 1.47
10	62.92 ± 0.98
15	68.5 ± 1.02
20	76.02 ± 0.76
25	78.62 ± 0.54

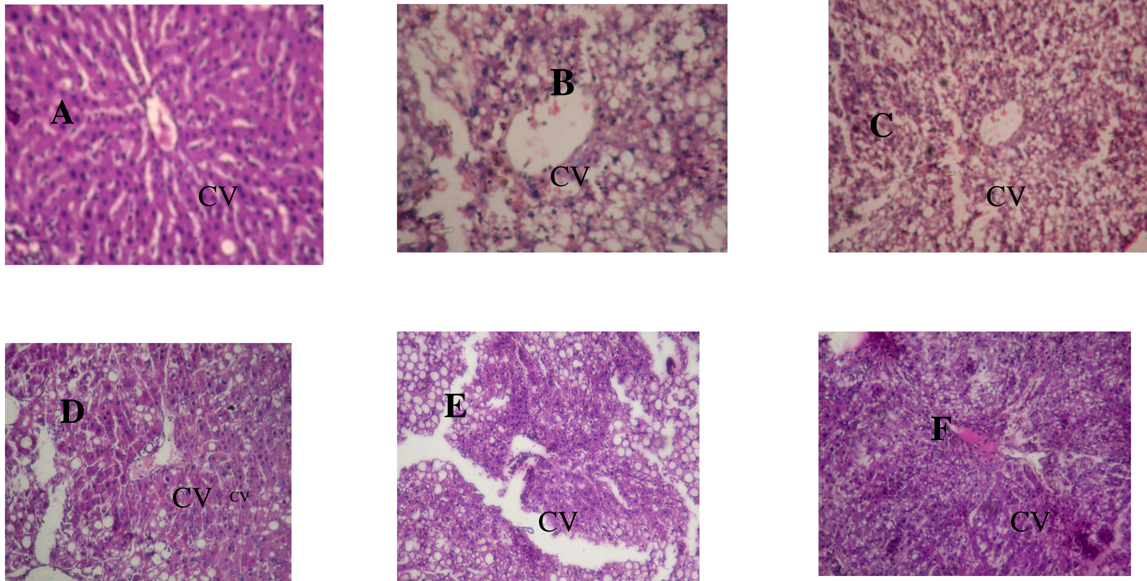

Fig. 1 Photomicrographs of liver section (haematoxylin-eosin)

Group I, x 200; (B) Group II, x 200 (CCl_4, 0.5 ml/ kg-.); (C) Group III, x 200 (CCl_4, 0.5 ml/ kg. + 100 mg /kg ARE);
(D) Group IV, x 100 (CCl_4, 0.5 ml kg^1. + 200 mg kg^{-1} ARE);
(E) Group V, x 100 (CCl_4, 0.5 ml kg^1 i.p. + 400 mg /kg^- ARE);
(F) Group VI, x 100 (CCl_4, 0.5 ml /kg + 2.5 ml /kg Liv-52) Fig.A: normal liver with normal hepatocytes

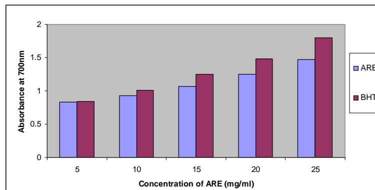

Fig 2. Reducing power of aqueous extract of root of *Tconnaroides* (ARE) and butylated hydroxy toluene (BHT) at different concentrate

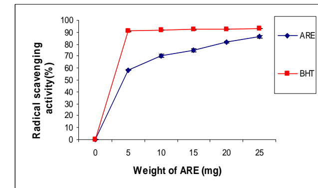

Fig 3. Radical scavenging ability of roots of *T. connaroides* (ARE) and butylated hydroxy toluene(BHT) at different concentrations.

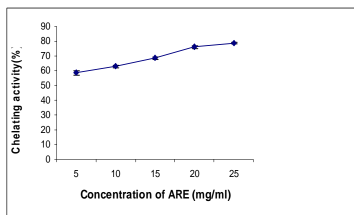

Fig 4 Chelating activity on Fe^{+2} of aqueous extract of *T. connaroides* (ARE) at different concentrations.

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