EVALUATION OF ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF *TERMINALIA CHEBULA* RETZ. ON CCL_4 INDUCED HEPATOTOXICITY IN RATS

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ABSTARCT

In the present study reported that antioxidant and hepatoprotective activities of the methanolic leaves extracts of *T. chebula*. From this study, methanolic leaves extract of showing the highest activity in the concentration of 500µg/ml (75.22±5.7 µM TEAC/mg DW). ABTS scavenging activity at various concentrations of the methanolic leaves extract of *T. chebula* showed the highest percentage inhibition ranged from 18.25±1.5 to 75.22±5.7 µM TEAC/mg DW. Methanolic leaves extract of *T. chebula* showed hepatoprotective activity in CCl_4 induced rats. The CCl_4 induced rats were significantly reduced ($p<0.05$) the body and liver weight when compared with control rats. The hepatic enzymes such as AST, ALT, ALP and GGT were significantly ($p<0.01$) increased CCl_4 treated group when compared with control. Methanolic leaves extracts of *T. chebula* with CCL_4 induced rats showed higher recovery hepatotoxic cells around portal tract. Silymarin treated group showed normal hepatocytes and their lobular architecture.

Key word: DPPH, antioxidant, CCl4, hepatoprotective and silymarin

INTRODUCTION

Plant medicines are great importance in the primary healthcare in many developing countries. Most of the medicinal plant parts are used as raw drugs and they possess varied medicinal properties (Mahesh and Sathish, 2008). Plants have a great potential for producing new drugs and used in traditional medicine to treat chronic and even infectious diseases (Panda *et al.,* 2009). Scientific evidence suggests that antioxidants reduce the risk for chronic diseases. Most of the antioxidant compounds are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. The main characteristic

of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms in nucleic acids, proteins, lipids or DNA and can initiate degenerative disease.

Liver injuries are a major worldwide health problem, with high endemicity in developing countries are mainly caused by chemicals and some drugs when taken in very high doses. Despite advances in modern medicine, there is no effective drug available that stimulates liver function, offer protection to the liver from damage or help to regenerate hepatic cells. Inflammatory disorders are another major health threat in the world. Drugs which are in use presently for the management inflammatory conditions are either narcotics (e.g. opioids) or nonnarcotic (e.g. salicylates) and corticosteroids (e.g. hydrocortisone).

Medicinal herbs provide protection against hepatotoxins in various ways: by enhancing the functioning of the hepatic glutathione antioxidant system (Ip and KO, 1996; Ip *et al.,*1996); inhibiting cytochrome P450, promoting glucuronidation, stimulating hepatic regeneration, activating functions of reticulo-endothelial systems, inhibiting biosynthesis of cyt P_{450}. (Rao and Mishra, 1998) preventing lipid peroxidation, stabilizing hepatocellular membrane, enhancing protein biosynthesis (Lin *el al.,* 1997); accelerating the regeneration of parenchymal cells and thus protecting against membrane fragility decreasing the leakage of marker enzymes into the circulation, interfering with the microsomal activation of $CC1_4$ and *i* or accelerating detoxification (Bishayee *et al.,*1995); counteracting the hepatic lysosomal enzymes (Saxena *et al.,*1993). Among the various numbers of plants are widely and abundantly distributed in this part of the world exhibit various biological and pharmacological activities. Only a few members have been studied for the pharmacological activities. In India, numerous medicinal plants and their formulations are used to combat liver diseases in traditional systems of medicine and folklore medicines (Asha and Pushpangadan, 1998). Hence the present study is aimed to investigate the antioxidant and hepatoprotective activities of methanolic leaves extract of *Terminalia chebula.*

MATERIALS AND METHODS

Collection of experimental plants

Healthy and young leaves of *Terminalia chebula* was collected distinctly from Thanjavur District, Tamil Nadu, India. The collected leaves were identified with help of the standard manuals such as "The Flora of the presidency of Madras" and Indian Medicinal plants. The leaves were separated from stems, washed in clean water, and dried at room temperature.

Preparation of plant extract

The collected leaves were cleaned and dried in shade for 7 days, then ground well to fine powder. About 500 g of each plants powder were extracted with methanol (80%) at 70°C by continuous hot percolation using soxhlet apparatus separately. The extraction was continued for 24 hrs. The methanolic extract was then filtered and kept in hot air oven at 40°C for 24 hrs to evaporate the methanol from it. A dark brown residue was obtained. The residue was kept separately in air tight containers and stored in a deep freezer (Harborne, 1984).

Antioxidant activity

DPPH Radical Scavenging Activity

Antioxidant reducing activity on DPPH radical was estimated according to the method of Blois (1958) with modification involving the use of high-throughput microplate system. Sample (50 μL of 1.0mg/mL) was added to 50 μL of DPPH (FG: 384.32) (1mM in ethanolic solution) and 150 μL of ethanol (absolute) in a 96-well microtiter plate in triplicates. The plate was shaken (15 seconds, 500 rpm) and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm. Ascorbic acid was used as positive control.

ABTS scavenging activity

The technique is based on the scavenging of $ABTS^{\cdot+}$ [(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt] radical cation which was generated by mixing solutions of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol/L) (Re *et al.,* 1999). The mixture was then incubated in the dark at room temperature for 16h. The product was diluted for optimal absorbance of 0.7 at 734 nm. The decolorization of the $ABTS^{\cdot+}$ solution by 100 µg/mL of the test

samples or reference compound (Trolox) was monitored by a decrease in absorption at 734 nm during 30 min. The antioxidant activity expressed in µM trolox equivalent antioxidant capacity (TEAC)/ mg dry weight.

FRAP assay

The method is based on reduction of ferric tripyridyltriazine (Fe3 – TPTZ) to ferrous complex tripyridyltriazine (Fe2 – TPTZ) by an antioxidant in acidic pH. The ferrous Fe (II) complex -TPTZ develops a blue color with maximal absorbance at 593 nm. The methodology of Benzie and Strain (1996) was used. FRAP (Ferric reducing antioxidant power) mixture consists of 10 parts of acetate buffer solution (300 mM) at pH 3.6, 1 volume of 10 mmol/l 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol/ l HCl and 1 volumes of a solution of FeCl3 6H2O (20 mM). To 2 ml of the FRAP mixture were added 10 µl of the plant extract. After incubation of 15 min at room temperature, the absorbance was measured at 593 nm. The calibration range was prepared with Trolox. Results are expressed as µmol Trolox equivalent antioxidant capacities (TEAC)/mg dry weight.

Hepatoprotectivity activity

Experimental Animals

Healthy wistar male albino rats, weighing about 150-220 g were obtained from Tamil Nadu Veterinary and Animal science college, Orathanadu, Thanjavur district, Tamil Nadu. Animals were maintained under standard conditions (12 h light / dark cycle; 25± 2ºC with 65 ± 5% humidity) and were fed with standard rat feed and water *ad libitum.* All the animals were acclimatized to laboratory conditions for a week before commencement of experiment. All the animals were followed the internationally accepted ethical guidelines for the care of laboratory animals. Prior to the experiments, animals were fed with standard food for one week in order to adapt to the laboratory conditions. All animal procedures were performed after approval from the institutional animal ethical committee (IAEC). (Reg.No. **KMCP/04/2016**).

Experimental conditions

The mice were divided in to seven groups comprising six animals in each group. The entire animals were injected with CCl_4 (1ml/kg/day p.o) except for the normal group as follows: Group 1: Control rat received normal saline (1ml/kg b.wt) orally for 21 days, Group 2: Negative

control rat received CCl_4 (1ml/kg/day b.wt) diluted with olive oil 1:1 ratio given by intraperitoneally for 21days, Group 3: Toxic control CCl_4 induced hepatotoxic rats' received standard drug Silymarin (10mg/kg b.wt) orally for 21 days, Group 4: CCl_4 induced rats received with methanolic leaves extract of *T. chebula* (200 mg/kg of b.wt) orally for 21 days. Silymarin and the plant extracts were administered concomitantly to the respective group of animals. Blood were collected by cardiac puncture and used to immediately.

Biochemical parameters

On 21th day, blood was collected through retro orbital vein and serum was separated by centrifugation at 2500 rpm for 10 minutes and stored at 4°C until use. The serum was used to estimate aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γ-GT), lactate dehydrogenase (LDH), total protein (Albumin/Globulin), bilirubin and lipid profile (Total cholesterol and Triglycerides). After collection of blood, the liver and kidney was immediately excised and rinsed in ice cold normal saline for further biochemical estimations. Determinations were done spectrophotometrically using Randox analytical kits according to standard procedures of manufacturer's protocols (Reitman and Frankel, 1957). From all the experimental groups, the portion of the liver was collected and rinsed with 0.15M phosphate buffer (pH 7.4) and centrifuged at 2000 rpm for 10 min at 4°C. The cell free supernatant was used for the estimation of lipid peroxide (LPO), glutathione peroxidase (GPx), reduced glutathione (GSH), super oxide dismutase (SOD), catalase (CAT) and TBARs, (Ellman, 1959).

Statistical analysis

The data acquired from animal trials were communicated as mean standard error (±S.E.M.). Statistical differences between the treatment and the control were assessed by ANOVA and Students–Newman–Keuls post hoc tests. Significance of data was expressed as $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

RESULTS

Antioxidant activity

Antioxidants are transfer, an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character. The antioxidant activity of DPPH free radical scavenging assay showed

the different plants extract. The scavenging activity of DPPH was increased at a concentration of 500 µg/ml, the scavenging ability on DPPH was 75.38±5.1% in methanolic leaves extract of *T. chebula*. The ABTS radical assay is also one of the most commonly used methods to evaluate the antioxidant activity. ABTS scavenging activity at various concentrations of the methanolic leaves extract of *T. chebula* showed the highest percentage inhibition ranged from 18.25±1.5 to 75.22±5.7 µM TEAC/mg DW. These results showed that varying solvent polarities differ significantly in their extraction capacity of antioxidant compounds, and therefore, their antioxidant activities. In the present study, the trends for ferric ion reducing activities of plant extracts were shown in Table. 1. The absorbance of methanolic leaves extract of *T. chebula* was clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration.

Hepatoprotectivity activity

The effect of *T. chebula* leaves extract on body and organ weight of control and experimental animals. The CCl_4 induced rats (Group 2) were significantly reduced ($p<0.05$) the body and liver weight when compared with control rats (Group 1). The body weight and organ weight were significantly increased in silymerin (162.5±1.62** g and 5.28±0.41g) and methanolic leaves extract of *T. chebula* (162.9±1.27*g) and (5.52±0.72*g) when compared to control group (Table.2). The hepatic enzymes such as AST, ALT, ALP and GGT were significantly ($p<0.01$) increased CCl_4 treated group when compared with control. The AST level was noted in the standard drug silymarin and *C. sinensis* were more or less both are equals. The ALT level was significantly reduced after treated with methanolic leaves extract of *T. chebula* (45.2±1.5 IU/L). The moderated level of ALP was significantly reduced with the treatment of *T. chebula* ($p<0.01$) (Table.3).Treatment with silymarin and methanolic leaves extract of experimental plants were significantly prevented the enzymatic changes in induced by CCl_4. The biochemical parameters such as total protein, albumin and globulin were significantly ($p<0.05$) decreased CCl_4 treated group (Gropup.2) when compared with control. The increased total protein content was recorded in the methanolic leaves extract of *T. chebula* (8.5±0.6* g/dl) (Table.4).

Antioxidant enzymes such as SOD, CAT and GPx activities were decreased in CCl_4 induced rats when compared with control. Among the plants, methanolic leaves extracts of *T. chebula* recorded the mostly increased level of SOD and GP_X. The Superoxide dismutase (SOD) level (3.90±2.21 U/gmHb) was decreased in CCl_4 intoxicated rats (Group.2) when compared with

control rats (Group.1). The CAT was mostly increased after treated with methanolic leaves extracts of *T. chebula.* The significant increased level GPx ($p<0.01$) also observed in the methanolic leaves extracts of *T. chebula.* The GSH level was also significantly increased ($p<0.01$) after treated with methanolic leaves extracts of *T. chebula* (Table.5). Rats are treated with CCl_4 was exhibited higher chlosterol ($223.0\pm1.8^{**}$ mg/dl) level compared to that control group ($130.5\pm1.5^{*}$ mg/dl). Methanolic leaves extracts of *T. chebula* were showed the significant reduction ($p<0.05$) of total cholesterol. Triglycerides level was decreased after treated with methanolic leaves extracts of *T. chebula* (159.2±3.5 mg/dl). LPO and TBARS level were significantly increased in group.2 (CCl_4 induced rats) when compared with other groups. In the present study, methanolic leaves extracts of *T. chebula* showed reduced level of LPO and TBARS (Table.6).

Table.1 Free radical scavenging activity of methanolic leaves extracts of *T. chebula*

Methanolic extracts (µg/ml)	DPPH (%)	ABTS (µM TEAC/mg DW)	FRAP (µM TEAC/mg DW)
100	18.25±1.2	18.25±1.5	46.54±2.1
200	43.32±2.3	43.32±2.3	49.22±4.3
300	64.71±3.5	64.71±3.5	54.23±5.4
400	68.04±4.2	65.04±4.2	58.45±6.3
500	75.38±5.1	75.22±5.7	65.52±7.2

Trolox equivalent antioxidant capacity (TEAC)

Table.2 Effect of methanolic extract of *T. chebula* **on body and organ weight in albino rats**

Experimental groups	**Body weight (g)**		**Liver weight (g)**
	Initial	**Final**	
Group 1	$157.2\pm1.12^{*}$	$164.6\pm1.14^{**}$	$5.9\pm0.45^{*}$
Group 2	$164.5\pm1.62^{*}$	$157.3\pm0.45^{*}$	$2.5\pm0.32^{*}$
Group 3	$168.4\pm1.83^{**}$	$162.5\pm1.62^{**}$	$5.28\pm0.41^{*}$
Group 4	$167.2\pm0.53^{*}$	$162.9\pm1.27^{*}$	$5.52\pm0.72^{*}$

Values are given as mean ± S.D (n=6). Values not sharing a common superscript letter significantly at *p<0.05 , **p<0.01, ***p<0.001

Table. 3 Effect of methanolic extract of *T. chebula* on liver marker enzymes of albino rats

Experimental groups	Liver marker enzymes			
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (IU/L)
Group 1	15.5±2.7*	32.4±5.2**	84.55 ± 0.60	42.0±3.2*
Group 2	98.9±4**	118.6±1.6**	105.2 ±0.04**	125.5±6.3**
Group 3	18.4±2.5**	37.8±2.5*	88.12 ± 0.02**	45.2±3.4*
Group 4	22.3±7.3*	45.2±1.5	92.08 ± 0.06**	56.8±2.3*

Values are given as mean ± S.D (n=6). Values not sharing a common superscript letter significantly at *p<0.05 , **p<0.01, ***p<0.001

Table. 4 Effect of methanolic extract of *T. chebula* on biochemical parameters of albino rats

Experimental groups	Biochemical parameters			
	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Bilirubin (g/dl)
Group 1	9.2±0.5*	6.2±0.1*	5.1±1.2	0.15±0.1*
Group 2	7.4±0.3*	5.5±0.3*	3.5±1.6*	1.28±1.7*
Group 3	9.5±0.8*	5.9±0.6*	4.7±3.1	0.17±0.5**
Group 4	8.5±0.6*	4.8±0.6*	4.4±0.6	0.21±0.7*

Values are given as mean ± S.D (n=6). Values not sharing a common superscript letter significantly at *p<0.05 , **p<0.01, ***p<0.001

Table. 5 Effect of methanolic extract of *T. chebula* on antioxidant enzymes of experimental rats

Experimental groups	Enzymatic antioxidants		
	SOD (U/gmHb)	CAT (U/gmHb)	GPx (U/gmHb)
Group 1	8.42± 0.73	24.90±0.55	32.33±0.48
Group 2	3.90±2.21	10.09±0.15	12.05±1.12
Group 3	7.12±1.45**	22.21±1.43**	30.98.1±0.16*
Group 4	6.25±1.43**	20.21±1.12**	29.31±3.12**

Values are given as mean ± S.D (n=6).Values not sharing a common superscript

letter significantly at *p<0.05 , **p<0.01, ***p<0.001

Table. 6 Effect of methanolic extract of *T. chebula* on lipid profile and lipid peroxidation of albino rats

Experimental groups	Lipid profile		Lipid peroxidation	
	Total cholesterol (mg/dl)	LPO (nmol/g)	LPO (nmol/g)	TBARS (nmol/ml)
Group 1	130.5±1.5*	4.5±0.2*	4.5±0.2*	1.38±0.10
Group 2	223.0±1.8**	8.1±0.1*	8.1±0.1*	3.18±0.42
Group 3	142.3±2.3**	5.7±0.6*	5.7±0.6*	1.77±0.15**
Group 4	158.4±1.5	5.8±0.2*	5.8±0.2*	1.90±0.12**

Values are given as mean ± S.D (n=6).Values not sharing a common superscript letter significantly at *p<0.05 , **p<0.01, ***p<0.001

DISCUSSION

Liver is the most vital organ concerned with the biochemical activity in the human body and it has great capacity to detoxicate toxic substances and synthesize useful metabolites (Meyer *et al.,* 2001). Chronic consumption of ethanol induces lipid peroxidation causing hepatotoxicity by increasing the free radical formation which in turn increases the level of lipid peroxide in hepatic tissue and causes cell injury (Lieber, 1991). DPPH assay is widely used to determine the antioxidant activity of plant extract. This assay is based on the ability of antioxidant compound to decolourise the purple colour of DPPH free radical in alcoholic solution to yellow colour (Premanath and Devi, 2011). In the present study, the methanolic leaves extract of *T. chebula* was showed the highest activity at 500 µg/ml concentrations. The $ABTS^{+}$scavenging assay, which employs a specific absorbance (734 nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples (Wu *et al.,*2006). The present study reported that the ABTS scavenging activity of the methanolic leaves extract of *T. chebula* showed the highest activity at 500 µg/ml concentration. In earlier report, the water and ethanol extracts of sumac (*Rhus.coriaria* L.) showed increased ferric reducing power with the increased concentration as standard antioxidants (Ercan and Ekrem. 2011). In this study also reported that the methanol leaves extract of *T. chebula* (65.52±7.2 µM TEAC/mg DW) were showed increased ferric reducing

power with the increased concentration (500µg/ml) and as compared with standard antioxidants (Table.1).

Liver damage is detected by the measurement of the activities of serum enzymes like AST, ALT, ALP and GGT, which has been released into the blood from damaged cells. They are also indicators of hepatic cell damage (Cay and Naziroglu, 1999). In the present study reported that the hepatic enzymes such as AST, ALT, ALP and GGT were significantly ($p<0.01$) increased CCl_4 treated group when compared with control. Hepatotoxins can interfere with the metabolism of bilirubin by: haemolytic reaction (i.e., increase in the load of conjugated bilirubin on liver cells by causing hemolysis); competing with serum albumin for binding with bilirubin or interference with the secretion of bilirubin by liver cell (Rao, 1973). In the present study, the biochemical parameters such as total protein, albumin and globulin were significantly ($p<0.05$) decreased CCl_4 treated group (Gropup.2) when compared with control. However, bilirubin was significantly increased in CCl_4 treated rats. The enzymatic antioxidant defence system is the nature protector against lipid peroxidation for important scavengers of superoxide ion and hydrogen peroxide (Dash *et al.,* 2007). In the present study, elevated level of LPO or TBARS in CCl_4 treated rats is a clear indication of exclusive formation of free radicals and activation of lipid peroxidation system. In earlier study reported that the enzymatic antioxidant defence system is the nature protector against lipid peroxidation for important scavengers of superoxide ion and hydrogen peroxide (Dash *et al.,* 2007).

CONCLUSION

Medicinal plants are widely used in management of diseases all over the world. Historically, the use of medicinal plants is as old as mankind and medicine. Our study identified the methanolic leaves extract of *T. chebula* was showed promising DPPH, ABTS and FRAP scavenging activity. In the present study also concluded that the experimental plants are involved protective role of liver damages. From this results can be concluded that the methanolic leaves extract of *T. chebula* have preventive effect against CCl_4 induced hepatocellular damage in rats. These result concluded that methanolic leaves extracts of *T. chebula* have potential for the development of new treatment against liver disease.

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