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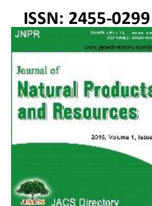

In Vitro Antioxidant and Hepatoprotective Effect of Leaf Galls of *Terminalia chebula* Retz., Against CCl_4 Induced Oxidative Stress in Liver Slice Culture Model

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ABSTRACT

The galls on *Terminalia chebula* Retz. (Family - Combretaceae) is a well-known herbal drug used to treat various diseases in Indian System of Traditional Medicine. The dried galls are widely commerce in the Indian market and substituted to galls of *Pistacia integerrima* Stew. Ex. Brandis. The present study aims to investigate antioxidant and hepatoprotective activity of hexane (TCH), ethanol (TCE) and water extract (TCW) of the galls on *T. chebula* by employing photochemiluminescence and spectrophotometric methods. The results showed that TCE and TCW significantly inhibited 2,2-diphenyl-1-picrylhydrazyl, nitric oxide and superoxide radical in dose dependent manner. Trent of phenol content was as: TCH < TCW < TCE. A significant correlation was shown by total phenol content and free radical scavenging activity of all extracts. The hepatoprotective effect was assayed in CCl_4-induced cytotoxicity in a liver slice culture model. The results revealed that significant depletion was observed in lactate dehydrogenase**,** lipid peroxidation, antioxidative enzymes superoxide dismutase, catalase, and Glutathione reductase on administration of the TCE and TCW or ascorbic acid as standard in the CCl_4-induced cytotoxicity in the liver. TCE and TCW extracts of galls on *T. chebula* have prevented significant oxidative liver damage.

1. Introduction

Terminalia chebula Retz. (Family - Combretaceae) is native to Southern Asia from India, Nepal, East to Southwestern China, South to Sri Lanka, Malaysia and Vietnam. The galls of *T. chebula* are produced by the plant due to an insect bite with or without an accompanying microorganism infection. The dried galls are widely commerce in the Indian market under name of 'Kakadshringi' as it is substituted to galls of *Pistacia integerrima* Stew. Ex. Brandis. (Family- Anacardiaceae) [1, 2].

It is widely used in Indian System of traditional medicine for treatment of diarrhea, dysentery, haemorrhoids, liver diseases, inflammation and for its rejuvenative effects. It is one of the components in 'Bal-Guti' and a suspension used to administer for healthy growth of the new born baby. It is considered very effective in pulmonary affections [3-6]. Antioxidant and whitening activities of phenolic compounds such as gallic acid, punicalagin, isoterchebulin, 1,3,6-tri-*O*-galloyl-*β*-D-glucopyranose, chebulagic acid and chebulinic acid from aqueous extract have been reported [6]. Pharmacognostic and phytochemical evaluation of leaf galls have been reported and the amount of gallic acid in the galls is 8.656 ± 0.024 mg/g [2]. Aquous extract at 0.1 mg/mL have been reported the highest 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity with scavenging of 84.64% and highest stimulation index (SI) on normal human fibroblast proliferation [6]. An aqueous extract of galls of *T. chebula* showed inhibitory effects on three dermatophytes and yeasts species [7]. Review of literature reveals that meager research work has been done on scientific validation of its claimed therapeutic uses. Reports regarding hepatoprotective effects of galls have not yet been documented. The present study deals with investigation of *in vitro* antioxidant employing photochemiluminescence and spectrophotometric methods and hepatoprotective activity of extracts of galls of *T. chebula* against carbon tetrachloride (CCl_4) induced oxidative stress in liver slice culture.

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2. Experimental Methods

2.1 Collection of Plant Material and Extraction

The galls on *Terminalia chebula* Retz. were collected from Satara (Mahabalewar), Maharashtra, India respectively in winter season of 2006-2007. The galls were authenticated and deposited in the crude drug repository of Agharkar Research Institute, Pune; vide voucher specimen numbers: AO 018.

The sample was shade dried, coarsely powdered and stored in an airtight container at 25 °C ± 4 °C. Powdered material (75 g) was extracted successively with hexane, ethanol and water using ASE 100 accelerated solvent extractor (Dionex, Vienna, Austria). Extraction was performed at 100 bar and temperature 60 °C for 20 min in the five replicate cycles. The extracts were concentrated under vacuum using rotary evaporator and yields of hexane (TCH), ethanol (TCE) and water extracts (TCW) were 2.15, 9.96 and 12.23 g respectively.

2.2 Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzo thiazoline-6-sulphonic acid) (ABTS), potassium persulphate and butylated hydroxytoluene (BHT) were obtained from Sigma Aldrich, Fluka, USA. Ethylene diaminetetraacetic acid (EDTA), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), monopotassium phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$), carbon tetrachloride (CCl_4), ascorbic acid, hydrochloric acid, potassium phosphate, glucose, folin-ciocalteu, other analytical grade solvents and regents were purchased from SD fine chemicals, Mumbai, India.

2.3 Antioxidant Activity

The abilities of the samples in scavenging DPPH radical were determined as per standard method [8]. Further, nitirc acid, superoxide radical and ABTS assays were performed according to reported method [9-11]. The method of photochemiluminescence (PCL) was used for determination of integral antioxidative capacity (IAC) of sample extracts. The equipment used was Photochem® with standard kit antioxidant capacity of water soluble compounds (AWL) and lipid soluble compound (ACL) (Analitikjena AG). Calibration and measurement were performed

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according to standard kit protocol. Total phenolic content in each extract was determined with folin-ciocalteu reagent [12] using pyrocatechol as a reference standard.

2.4 In vitro Hepatoprotective Activity

2.4.1 Animals

To assess the hepatoprotective activity, adult albino mice (6–8 weeks old, weight 25-30 g) of either sex breed in the animal house of Agharkar Research Institute, Pune, were used for the preparation of liver slices. The approval for this work, using animals, was taken from the Institutional Animal Ethical Committee of Agharkar Research Institute, Pune.

2.4.2 Liver Slice Culture

Liver slice culture was maintained according to reported protocol [13, 14]. The mice were dissected open after cervical dislocation, and liver lobes were removed and transferred to pre-warmed Kred's Ringer Hepes (KRH) (2.5 mM Hepes, pH 7.4, 11.8 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM β-hydroxy butarate and 4.0 mM glucose). The liver was cut into thin slices using sharp blade. The slices were weighed and the slices weighing between 4 and 6 mg were used for the experiment. Each experimental system contained 20-22 slices weighing together 100-120 mg. These slices were washed with 10 mL KRH medium, every 10 min over a period of 1 h. These were then pre-incubated for 60 min in small plugged beakers containing 2 mL KRH on a shaker water bath at 37 °C. At the end of pre-incubation, the medium was replaced by 2 mL of fresh KRH and incubated for 2 h at 37 °C.

2.4.3 Experiment Design

The liver slices were further divided into individual cultures for the further respective treatments. Set 1, control, slices incubated in KRH medium; set 2, slices incubated in 15.5 mM CCl_4; set 3, slices incubated in 25 μg/mL TCH; set 4, slices incubated in 25 μg/mL TCE; set 5 slices incubated in 25 μg/mL TCW; set 6, slices incubated in 15.5 mM CCl_4 + different concentration (5, 10, 25, μg/mL) of TCH; set 7, slices incubated in 15.5 mM CCl_4 + different concentration (5, 10, 25, μg/mL) of TCE; set 8, slices incubated in 15.5 mM CCl_4 + different concentration (5, 10, 25, μg/mL) of TCW; set 9, slices incubated in 15.5 mM CCl_4 + 10 mM ascorbic acid; set 10, slices incubated in 10 mM ascorbic acid.

After the respective treatments, all the cultures were incubated in constant temperature water bath at 37 °C for 2 h. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg mL^{-1}. The culture medium was collected and used for estimation of lactate dehydrogenase (LDH), which was employed as a cytotoxicity marker. The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatants assayed for LDH, catalase, peroxidase and superoxide dismutase. Ascorbic acid (AA) was used as standard. All assays were measured by following the instructions on commercial kits (Accurex Biomedical Pvt. Ltd. India). Lipid peroxidation was determined in terms of malondialdehyde (MDA). Ascorbic acid (AA) was used as standard.

2.5 Statistical Analysis

Data were expressed as mean ± standard deviation. The results of treatment effects were analyzed using one-way ANOVA test (Graphpad Prism 4) and p values < 0.001 were considered as very significant and p values < 0.05 were considered as significant.

3. Results and Discussion

3.1 Antioxidant Activity

Antioxidant activity was evaluated in terms of scavenging of DPPH, nitric oxide SO radical and TEAC, photo-chemiluminescence *in vitro* systems. The DPPH radical scavenging assay is commonly employed to evaluate the ability of antioxidants to scavenge free radicals. In the present study, TCW and TCE showed high antioxidant activity followed TCH which significantly decreased the DPPH radical as compared to standard (Fig. 1). IC_{50} values of TCH, TCE and TCW were 26.35 ± 2.45, 20.67 ± 1.89 and 28.49 ± 2.34 μg/mL, respectively. IC_{50} value of positive control ascorbic acid was 4.1 μg/mL.

Sodium nitroprusside (SNP) is known to decompose in aqueous solution at physiological pH (7.2) producing NO. SNP spontaneously releases nitric oxide (NO) in solution and the amount of NO released can be inferred by using the Griess' reagent. This reagent reacts with nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO, and therefore allows an indirect estimation of the amount of NO released in the solution [9, 15]. TCH had low nitric oxide inhibition activity than TCE and TCW and activity was dose dependent (Fig. 1). IC_{50} values of TCH, TCE and TCW were 28.53 ± 1.85, 19.66 ± 1.73 and 31.34 ± 1.67 μg/mL, respectively. IC_{50} value of standard ascorbic acid was 3.9 μg/mL.

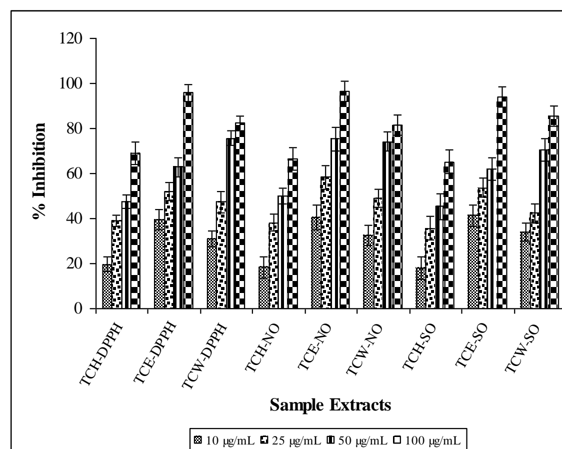

Fig. 1: Free radical scavenging capacity of leaf galls of *T. chebula* extracts (Values are mean ± SD of three experiments)

Superoxide radical $O_2^{-\cdot}$ is a highly toxic species, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyze the breakdown of $O_2^{-\cdot}$ radical [15]. The potassium superoxide assay was used to measure the superoxide dismutase activity of extracts. TCH showed low superoxide dismutase scavenging activity than TCE and TCW and activity was dose dependent (Fig. 1). IC_{50} values of TCH, TCE and TCW were 77.74 ± 1.65, 18.66 ± 2.33 and 32.34 ± 2.87 μg/mL, respectively. IC_{50} value of ascorbic acid was 5.3 μg/mL.

Peroxyl radicals or other oxidants like potassium persulphate oxidize ABTS to its radical cation, $ABTS^+$. The antioxidant capacities were determined by measuring decrease in the intensity of the blue colour as a result of reaction between the $ABTS^+$ radical [15] and the antioxidant compounds in the sample. Trend of TEAC values was as TCE > TCW > TCH (Table 1).

Table 1 Free radical scavenging capacity of leaf galls of *T. chebula* extracts

Samples	TEAC (mM)	Photochemiluminescence ACW in ascorbic acid equivalent (nmol/g)	Photochemiluminescence ACL in trolox equivalent (nmol/g)
TCH	8.47±0.06	12.213	8.342
TCE	3.89±0.19	2.641	8.686
TCW	3.58±0.193	5.541	13.690
AA	3.89±0.23	-	-

Values are mean ± SD of three experiments

Photochem® apparatus allowed precise method for the integral antioxidative capacity. Free radicals are generated in the instrument by means of photosensitizer and detected by their reaction with a chemiluminogenic substance [16]. Calibration curves of standard ascorbic acid and trolox were determined for the calculation of ascorbic acid and trolox equivalents of ACW and ACL. All sample extract had distinctly varied ACW and ACL values. TCE and TCW had high antioxidant capacity than TCH (Table 1).

The key role of phenol compounds is the ability to scavenge free radicals and ROS such as singlet oxygen, superoxide free radical, and hydroxyl radicals. Phenol compounds in the medicinal plants extracts are frequently responsible for the antioxidant status, thus total phenol content has been determined [17]. The trade of phenol content was as: TCH (84 mg PCE/g) < TCW (234 mg PCE/g) < TCE (289 mg PCE/g). A significant correlation was shown by total phenol content and free radical scavenging activities of all extracts. The results exhibited that greater antioxidant activity of TCE and TCW may be due to their highest amount of phenol compounds.

3.2 In Vitro Hepatoprotective Activity

The liver slice is a microcosm of the intact liver consisting of highly organized cellular community in which different cell types are subject to mutual contact. Such culture offers analysis of hepatotoxic events by measuring the release of LDH into the medium. Therefore, liver slice culture model is an *in vitro* technique that offers the advantages of *in vivo* as it provides desirable complexity of structurally and functionally intact

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cells [18, 19]. It provides valuable approaches for screening of plant extracts/fractions for their hepatoprotective activity and elucidation of possible mechanism of actions.

Oxidative stress was induced by adding cytotoxic CCl_4 to the liver slice culture. Release of LDH in the liver slice culture medium was used as cytotoxicity marker. CCl_4 was highly toxic to the treated cells which increased LDH concentration in the medium as compare to control. The TCH, TCE and TCW were found to be non-toxic at dose 25 µg/mL. TCH (7.00±2.09 units/100 mg tissue wet wt.), TCE (7.33±1.16 units/100 mg tissue wet wt.) and TCW (7.17±1.47 units/100 mg tissue wet wt.) treated liver slices showed releases of LDH percentage was found to be similar to that of control untreated slices (7.56±1.16 units/100 mg tissue wet wt.). In all further experiments all extracts at dose of 25 µg/mL were used.

The LDH release in the culture system treated with CCl_4 was 6 times more (43.83±1.63 units/100 mg tissue wet wt.) as compared to control. The amount of LDH release in medium reduced very significantly ($p<0.001$) after addition of TCE and TCW than TCH ($p<0.05$) along with CCl_4 cytotoxicant. The activity was comparable with ascorbic acid used as standard (Table 2).

Table 2 Effect of extracts in protecting liver cells from CCl_4 induced cytotoxicity by ameliorating oxidative stress

Treatments	LDH Units/100 mg tissue wet wt.	SOD Units/100 mg tissue wet wt.	CAT Units/100 mg tissue wet wt.	GR Units/100 mg tissue wet wt.
Control	7.56±1.16	17.34±1.47	14.00±1.41	0.141±0.012
15.5 mM CCl_4	43.83±1.63	56.33±2.75	86.5±1.04	0.488±0.008
AA	7.53±1.47	17.5±2.34	14.33±1.75	0.140±0.007
GH	7.00±2.09	17.43±1.82	14.65±1.37	0.146±0.003
GE	7.33±1.16	17.23±1.49	14.83±1.22	0.149±0.011
GW	7.17±1.47	17.45±1.41	14.67±1.67	0.148±0.006
15.5 mM CCl_4 + TCH 5 µg/mL	37.33±0.81[a]	46.67±0.81[a]	43.83±0.75[a]	0.346±0.055[a]
15.5 mM CCl_4 + TCH 10 µg/mL	30.0±0.89[a]	43.0±0.89[a]	38.5±0.54[a]	0.326±0.005[a]
15.5 mM CCl_4 + TCH 25 µg/mL	26.0±0.89*	40.17±1.94*	35.67±0.81*	0.288±0.008*
15.5 mM CCl_4 + TCE 5 µg/mL	27.83±0.75[a]	29.83±0.75[a]	39.5±1.37[a]	0.272±0.008[a]
15.5 mM CCl_4 + TCE 10 µg/mL	24.33±2.48*	24.5±0.54*	28.24±1.09*	0.236±0.008*
15.5 mM CCl_4 + TCE 25 µg/mL	17.83±1.21*	18.17±1.16*	22.74±2.28*	0.206±0.008*
15.5 mM CCl_4 + TCW 5 µg/mL	30.33±1.04[a]	31.0±0.89[a]	43.67±1.03[a]	0.312±0.008[a]
15.5 mM CCl_4 +TCW 10 µg/mL	26.5±1.09*	25.33±1.96*	33.5±1.04*	0.284±0.005*
15.5 mM CCl_4 +TCW 25 µg/mL	19.33±1.36*	19.67±0.81*	25.17±2.13*	0.251±0.007*
15.5 mM CCl_4 + 50 mM AA	12.67±1.03*	15.33±1.63*	19.83±1.83*	0.189±0.018*

Cytotoxicity was assessed in terms of % lactate dehydrogenase (LDH) released, and the response to oxidative stress was measured in terms of antioxidant enzymes SOD, superoxide dismutase; CAT, Catalase; GR, Glutathione reductase activity. Ascorbic acid was used as a standard.

TCH, Hexane extract, TCE, Ethanol extract and TCW, Water extract.

Values represent means of at least three experiments and their standard deviation.

[a] Significant differ compared with respective CCl_4 treated group, $p < 0.05$

* Significant differ compared with respective CCl_4 treated group, $p < 0.001$

CCl_4 is known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation in liver tissue. The lipid peroxidation levels in the liver slice culture medium were assessed by TBARS assay. Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances and was expressed as µmol of malondialdehyde formed/100 mg tissue. The amount of lipid peroxidation increased folds in CCl_4 (Fig. 2) treated liver cells compared to respective control. The extent of lipid peroxidation was reduced to near control levels very significantly ($p<0.001$) when liver cells were treated either with TCE and TCW than TCH along with CCl_4 (Fig. 2).

Time course of lipid peroxidation was assessed in the presence of cytotoxic agent alone and together with different extracts. CCl_4 treated cells showed increase in lipid peroxidation paralleled with the increase in LDH release by the cells. However, in presence of TCE and TCW along with cytotoxic agent the lipid peroxidation, like the LDH release, returned to the control levels which was very significant ($p<0.001$) (Fig. 2) than TCH. Since lipid peroxidation is caused by free radicals, all extracts appears to reduce the amount of free radicals substantially.

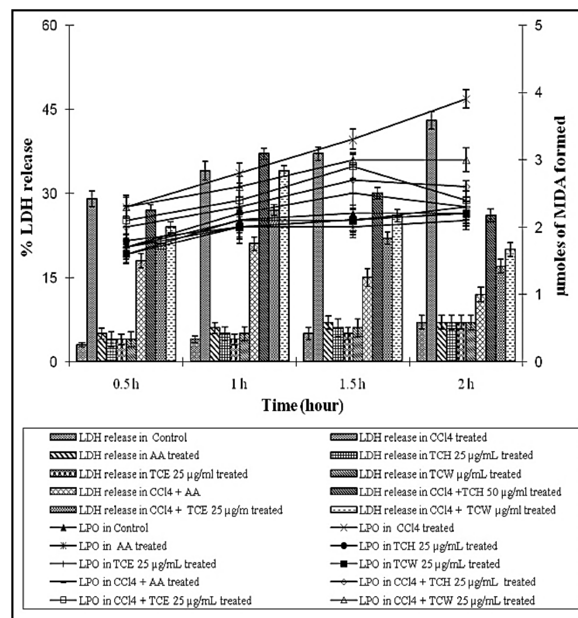

Fig. 2 Percentage release of LDH and extend of lipid peroxidation in liver slice culture in CCl_4 induced cytotoxicity (Values are mean of three experiments; CCl_4 – Carbon tetrachloride; AA, Standard ascorbic acid, at 50 mM concentration; LPO, Lipid perioxidation; TCH, Hexane extract, TCE, Ethanol extract and TCW, Water extract)

CCl_4 induces oxidative stress in the cells by generation of ROS. Antioxidant enzymes (AOEs) SOD, CAT, and GR protect cells from oxidative stress of highly reactive free radicals. Oxidative SOD and CAT are known enzymes to prevent damage by directly scavenging the harmful active oxygen species. GR plays a role in recycling the oxidized glutathione to reduced glutathione, which acts as an antioxidant [19]. Activities of all three AOEs were checked in liver slice culture treated with CCl_4 alone or CCl_4 and extracts. The activities of SOD, CAT, and GR were increased in the liver tissue treated with CCl_4. The liver tissue treated with TCE and TCW along with CCl_4 showed very significant ($p<0.001$) reduced antioxidant enzymes activities when added along with the toxicants to the culture (Table 2).

The results in this study revealed that significant depletion was observed in the lipid peroxidation, antioxidative enzymes SOD, CAT, and GR on the administration of the TCE and TCW or ascorbic acid in the CCl_4 induced toxicity in the liver.

4. Conclusion

We report for the first time, *in vitro* hepatoprotective activity of extracts of galls on *Terminalia chebula*. The results of present study suggest that the treatment with hexane and ethanol extracts was effective against CCl_4 induced hepatic damage in liver slice culture model. The strong hepatoprotective activity shown by the hexane and ethanol extracts may be attributed to its free radical scavenging capacity, inhibition of lipid peroxidation, increase in antioxidant enzymes SOD and CAT and non-enzymatic antioxidant GSH. Further comprehensive pharmacological investigations will be needed to validate the ethnobotanical claims and elucidate the mechanism of hepatoprotective activity.

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