In Vitro Antioxidant and Hepatoprotective Effect of Leaf Galls of Terminalia chebula Retz., Against CCl₄ Induced Oxidative Stress in Liver Slice Culture Model

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ARTICLE DETAILS

Article history:
Received 23 September 2015
Accepted 30 September 2015
Available online 01 October 2015

Keywords:
Terminalia chebula Retz.
Oxidative Stress
Liver Slice Culture Model

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 under name of ‘Kakadshringi’ as it is 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 < TCE < TCW. A significant correlation was shown by total phenol content and free radical scavenging activity of all extracts. The hepatoprotective effect was assayed in CCl₄-induced cytotoxicity in a liver slice culture model. The results revealed that significant depletion was observed in lactate dehydrogenase, lipid peroxidation, antioxidant enzymes superoxide dismutase, catalase, and Glutathione reductase on administration of the TCE and TCW as acaric acid as standard in the CCl₄-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. 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 < TCE < TCW. A significant correlation was shown by total phenol content and free radical scavenging activity of all extracts. The hepatoprotective effect was assayed in CCl₄-induced cytotoxicity in a liver slice culture model. The results revealed that significant depletion was observed in lactate dehydrogenase, lipid peroxidation, antioxidant enzymes superoxide dismutase, catalase, and Glutathione reductase on administration of the TCE and TCW as acaric acid as standard in the CCl₄-induced cytotoxicity in the liver. TCE and TCW extracts of galls on T. chebula have prevented significant oxidative liver damage.

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: A0 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-ethylbenzthiazoline-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 (CaCl₂), monopotassium phosphate (KH₂PO₄), magnesium sulphate (MgSO₄), carbon tetrachloride (CCl₄), acetic acid, hydrochloric acid, potassium sulphate, 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, nitric 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
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 fasted open after cervical dislocation, and liver lobes were removed and transferred to pre-warmed Kreb’s Ringer Hepes (KRH) (2.5 mM Hepes, pH 7.4, 11.8 mM NaCl, 2.85 mM KCl, 2.5 mM CaCl2, 1.5 mM KH2PO4, 1.18 mM MgSO4, 5 mM β-hydroxy buturate and 4 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 consisted of 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 sealers 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 CCl4; set 3, slices incubated in 15.5 mM CCl4 + 25 mM ascorbic acid and trolox. Ascorbic acid (AA) was used as standard. 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. 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). IC50 values of TCH, TCE and TCW were 28.53 ± 1.85, 19.66 ± 1.73 and 31.34 ± 1.67 µg/mL, respectively. IC50 value of standard ascorbic acid was 3.9 µg/mL.

![Free radical scavenging capacity of leaf galls of T. chebula extracts](image)

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

Superoxide radical O2− 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 O2− 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). IC50 values of TCH, TCE and TCW were 77.74 ± 1.65, 18.66 ± 2.33 and 32.34 ± 2.07 µg/mL, respectively. IC50 value of ascorbic acid was 5.3 µg/mL.

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>TEAC (nmol/mL)</th>
<th>Photochemiluminescence in trolox equivalent (nmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCH</td>
<td>8.47 ± 0.06</td>
<td>12.213</td>
</tr>
<tr>
<td>TCE</td>
<td>3.89 ± 0.19</td>
<td>5.541</td>
</tr>
<tr>
<td>TCW</td>
<td>3.58 ± 0.19</td>
<td>13.690</td>
</tr>
<tr>
<td>AA</td>
<td>3.89 ± 0.23</td>
<td>-</td>
</tr>
</tbody>
</table>

Table: 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 chemiluminescent substance [16]. Calibration curves of standard ascorbic acid and trolox were determined for the calculation of trolox equivalent of ACW and ACL. All sample extracts 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 plant 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) > TCE (234 mg PCE/g) > TCW (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

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

Oxidative stress was induced by adding cytotoxic CCl<sub>4</sub> to the liver slice culture. Release of LDH in the liver slice culture medium was used as cytotoxicity marker. CCl<sub>4</sub> was highly toxic to the treated cells which increased LDH concentration in the medium as compare to control. The TCE and TCW were found to be non-toxic at dose 25 µg/mL. TCH (7.06±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 each extracts at dose of 25 µg/mL were used. The LDH release in culture system treated with CCl<sub>4</sub> 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<sub>4</sub> and cytoxicant. The activity was comparable with ascorbic acid used as standard (Table 2).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LDH</th>
<th>SOD</th>
<th>CAT</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units/100</td>
<td>Units/100</td>
<td>Units/100</td>
<td>Units/100</td>
</tr>
<tr>
<td>mg tissue wet wt.</td>
<td>mg tissue</td>
<td>mg tissue</td>
<td>mg tissue</td>
<td>mg tissue</td>
</tr>
<tr>
<td>Control</td>
<td>7.56±1.16</td>
<td>17.3±1.47</td>
<td>14.00±1.41</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>15.5 mM CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>43.83±1.63</td>
<td>53.6±2.75</td>
<td>86.5±1.04</td>
<td>0.48±0.008</td>
</tr>
<tr>
<td>AA</td>
<td>7.53±1.47</td>
<td>17.5±3.24</td>
<td>14.00±1.75</td>
<td>0.14±0.007</td>
</tr>
<tr>
<td>TCH</td>
<td>0.00±2.09</td>
<td>17.4±3.18</td>
<td>16.4±1.37</td>
<td>0.14±0.003</td>
</tr>
<tr>
<td>TCW</td>
<td>7.33±1.16</td>
<td>17.2±3.14</td>
<td>14.8±1.22</td>
<td>0.14±0.011</td>
</tr>
<tr>
<td>GW</td>
<td>7.17±1.47</td>
<td>17.45±1.41</td>
<td>1.67±1.67</td>
<td>0.14±0.006</td>
</tr>
</tbody>
</table>

The amount of LDH release in medium reduced very significantly (p<0.001) after addition of CCl<sub>4</sub> and cytoxicant. The activity was comparable with ascorbic acid used as standard (Table 2).

Cytotoxicity was assessed in protective liver cells from CCl<sub>4</sub> induced cytotoxicity by ameliorating oxidative stress

### 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<sub>4</sub> 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 elucidate the ethnomedical claims and elucidate the mechanism of hepatoprotective activity.

### Acknowledgments

The authors are greatly thankful to the Director and In-Charge Biodiversity and Palaeobiology, Agharkar Research Institute, Pune, India for providing facilities and encouragement throughout the work.

### References


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