Hepatoprotective and Free Radical Scavenging Activities of Extracts and a Major Compound Isolated from the Leaves of *Cineraria abyssinica* Sch. Bip. ex A. Rich.

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**ABSTRACT**

In Ethiopian traditional medicine the aqueous decoction of the leaves of *Cineraria abyssinica* Sch. Bip. ex A. Rich (Asteraceae) is used for the treatment of various ailments including liver diseases, however, to date, there appears to have been no scientific report on the phytochemistry and claimed hepatoprotective activity of the plant. The main purpose of this study was, therefore, to carry out hepatoprotective and antioxidant activities of the leaf extracts of *C. abyssinica*. Hepatoprotective activities of the aqueous and 80% methanolic extracts as well as the methanol fraction of the leaves of *C. abyssinica* were investigated against carbon tetrachloride-induced liver damage in rats. Intraperitoneal administration of 2 ml/kg of CCl₄ (50% in liquid paraffin) significantly (p < 0.001) raised the plasma levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the toxin group compared with the values in the control group. Pretreatment of rats with 200 mg/kg of the aqueous, 80% methanol extracts and the methanol fraction reduced the toxin-induced rise in plasma ALP (65%, 75.4%, 85%), ALT (46.1%, 42.3%, 75%), and AST (58%, 98%, 79%), respectively. The standard drug, silymarin (100 mg/kg) reduced serum ALP (88%), ALT (92%), and AST (87.3%). Bioactivity-guided fractionation of the methanol fraction resulted in the isolation of the flavonol glycoside rutin, whose structure was assigned on the basis of spectroscopic methods. The results of biochemical analysis were further verified by histopathological examination of the liver, which showed improved architecture, absence of necrosis and a decrease in inflammation, compared with the findings in the toxin group of animals. Both the extracts and rutin showed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities. Acute toxicity studies showed that the total extracts of the plant are nontoxic up to a dose of 3 g/kg. The present study revealed for the first time the presence of a hepatoprotective and antioxidant phytochemical in the leaves of *C. abyssinica* that scientifically validates the traditional use of the plant and its potential for the treatment of liver disorders.

**Key words:** *Cineraria abyssinica*, Asteraceae, hepatoprotective, rutin, free radical scavenging

**INTRODUCTION**

Liver disease is one of the major causes of morbidity and mortality in public, affecting humans of all ages throughout the world. Despite the great stride in allopathic medicine, modern drugs available for liver diseases have so many limitations. They are limited in number, they do not provide a complete cure and they are unaffordable to most people in the developing countries. This situation stresses the importance of worldwide public–private partnerships to enhance the research enterprise, bring new agents to market in a more cost-effective fashion, and provide effective therapies to suffering patients at costs that are within their reach.1–3

Traditional medicines continue to provide front-line pharmacotherapy for many millions of people worldwide. In the absence of safe and reliable antihapatotoxic modern drugs, several medicinal plants have been used worldwide in various traditional herbal recipes for the prevention and...
treatment of liver disease. In recent years there has been a growing focus to follow systematic research methodology and to scientifically evaluate the basis for traditional herbal medicines which are claimed to possess hepatoprotective activity.[1,4]

*Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) commonly known by its vernacular name ‘*Etsemefirb*’, is an erect or scrambling, annual or perennial herb that can grow up to 20-100 cm high. It has repeatedly branched stem, with alternate, simple to lyrate pinnatifid petiolate leaves and radiate capitula with yellow florets. It extends from Ethiopia into Yemen and Saudi Arabia.[3] Based on the information provided by the traditional community from Harar, eastern part of Ethiopia, the aqueous decoction of the leaves of *C. abyssinica* is employed for the treatment of various ailments such as hypertension, cancer, diabetes, diarrhea, kidney and liver diseases. However, despite its wider use in traditional medicine, there are no prior reports on the phytochemistry and pharmacological effects of this plant. The present research was therefore, undertaken to examine the possible hepatoprotective action of the plant using *in vivo* CCl₄-induced hepatotoxicity test in rats and to examine its *in vitro* DPPH free radical scavenging effect.

**MATERIALS AND METHODS**

**Plant material**

The leaves of *C. abyssinica* were collected from and around the town of Harar in the Harari People Region, 525 km East of Addis Ababa, Ethiopia in September 2008. The plant was authenticated by Ato Melaku Wondafrash of the National Herbarium, Addis Ababa University, where a voucher specimen has been deposited (Collection number, B 01).

**Animals**

Wistar albino male rats (200-250 g) and mice (25-30 g) obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) animal house were used for the experiments. The animals were housed under standard laboratory conditions and were fed commercial rat feed and tap water *ad libitum*. The animals were fasted overnight with free access to water and acclimatized for one week in the new environment before experiments were carried out. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline[8] and approved by the Institutional Review Board of the School of Pharmacy, Addis Ababa university.

**Chemicals and instruments**

All the chemicals and reagents used for the experiments were analytical grade. Ultraviolet (UV) spectra were run on a Shimadzu UV-1800 spectrophotometer. Infra red (IR) spectra were taken on a Shimadzu IR Prestige-21 spectrophotometer in KBr pellets. ¹H NMR and ¹³C NMR spectra were recorded in DMSO-d₆ using a Bruker A-400 spectrometer with TMS as an internal standard. Electro spray mass spectra were obtained with LCQ Deca XP, ESI, negative mode spectrometer.

**Preparation of crude extracts**

A hydroalcoholic extract of *C. abyssinica* was prepared by macerating 300 g of the powdered shade-dried leaves with 80% methanol (3x, each for 72 h) with occasional shaking. The combined filtrates were then dried in a rotary vacuum evaporator at a temperature not exceeding 40°C. Aqueous extract was prepared by boiling the plant material for 30 min followed by cooling, filtering and lyophilizing of the extract.

**Preparation of solvent fractions and isolation of a compound**

The air-dried powdered leaves of *C. abyssinica* (300 g) were successively extracted in a Soxhlet apparatus using solvents of increasing polarity, starting from chloroform then acetone and methanol. The solvents were removed using a rotary vacuum evaporator at a temperature not exceeding 40°C. The most active methanol fraction was subjected to silica gel preparative thin layer chromatography (PTLC) using butanol: acetic acid: water (4:1:5, upper phase) as a mobile phase. The yellowish powder obtained was further purified by LH-20 column chromatography using methanol as solvent and the purity of the eluate was checked by analytical TLC.

**Identification of the isolated compound**

The isolated compound was identified as rutin by comparison of its spectral data (¹H and ¹³C-NMR) with those reported in the literature.[7,8] Furthermore, comparison of the ESI-mass spectra of the isolated compound was found to be superimposable on those of standard rutin.

**Acute toxicity tests**

Acute toxicity studies were carried out on the aqueous and 80% methanolic leaf extracts of *C. abyssinica* according to Daisya *et al*.[5] Normal healthy male mice fasted for 12 h were randomly divided into drug-treated ‘test’ groups and vehicle-treated ‘control’ group, of 6 mice per group. Each of the extracts (0.5, 2.0 and 3.0 g) suspended in 1% carboxyl methyl cellulose (CMC) was separately administered orally to the mice in each of the test groups. The mice in the control group were treated with vehicle alone (1% CMC). Two h after treatment, the mice in both the test and control groups were given free access to food and water, and behavioral changes were observed over a period of 24 h. Mortality, if any, caused by the extract within this period of time was also observed.
In vivo hepatoprotective activity studies

The model described by Narayan et al.\textsuperscript{10} was employed. The rats were divided into seven groups consisting of five animals each. Animals in group A served as normal and they were given only vehicle (0.7% CMC suspension 1 ml/kg b.w.) orally for 6 days. Animals in group B served as toxin control and they were administered with CCl\textsubscript{4} (50% solution of CCl\textsubscript{4} in liquid paraffin, 2 ml/kg b.w., i.p.) on the 4\textsuperscript{th} day and with vehicle on rest of the days. Animals in groups C-E were treated orally with 200 mg/kg of the aqueous extract, the 80% methanolic extract and the methanol fraction, respectively, suspended in 0.7% CMC for 6 days and CCl\textsubscript{4} (2 ml/kg b.w.) on the 4\textsuperscript{th} day i.p, while the animals of groups F and G received rutin and silymarin (100 mg/kg b.w. suspended in 0.7% CMC), respectively, for 6 days orally and CCl\textsubscript{4} (2 ml/kg b.w.) on the 4\textsuperscript{th} day i.p. On the 7\textsuperscript{th} day, the animals were anesthetized by ether and blood was collected in heparinized tubes from the retro orbital plexus of each animal and serum was separated by centrifugation at 365 rpm for 15 min and analyzed for various biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

Histopathological study

Immediately after collection of blood from each animal, they were sacrificed by cervical dislocation and the liver was separated, washed with normal saline and stored in 10% formalin. Small pieces of the liver fixed in 10% formalin, were processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with hematoxylin and eosin (H & E) and examined under the microscope for histopathological changes. Images were captured using Olympus DP12 CCD camera at original magnification of 400×.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity test

The method of Sokmen et al.\textsuperscript{11} was used in this experiment. Firstly, 5 ml of 0.004% DPPH in methanol was mixed with 50 µl of various concentrations (1000, 500, 250, 125, 50, µg/ml) of the crude extracts, fractions, the isolated compound or ascorbic acid (a reference compound) separately. Following 30 min of incubation at room temperature in the dark, the absorbance of the mixture in the samples was measured using a spectrophotometer (Unico\textsuperscript{12} 2100) at 517 nm against methanol as blank. Percentage radical scavenging activity of the samples was evaluated by comparing with a control (5 ml DPPH solution and 50 µl methanol). Each sample was measured in triplicate and averaged. The percentage radical scavenging activity (RSA) was calculated using the following formula:

\[
\text{RSA} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where, \(A_0\) is absorbance of the control, and \(A_1\) is absorbance of samples after 30 min. Free radical scavenging activities of the crude extracts, fractions, rutin and ascorbic acid were expressed as IC\textsubscript{50}. The IC\textsubscript{50} value was defined as concentration (in µg/ml) of sample that inhibits 50% of the formation of DPPH radical.

Data and statistical analysis

Data are expressed as mean ± SD (standard deviation). Statistical evaluation of data was performed by using one-way analysis of variance (ANOVA) and Turkey post test. Values were considered to differ significantly for P < 0.01 to P < 0.001. The % protection of the test material was calculated by the following formula:

\[
\text{% Protection} = \left( \frac{(a-b)}{(a-c)} \right) \times 100
\]

where, a is the mean value of the marker produced by hepatotoxin; b is the mean value of the marker produced by toxin plus test material; and c is the mean value produced by the vehicle control.

RESULTS AND DISCUSSION

Acute toxicity

In the present study, a preliminary toxicity study was designed to demonstrate the appropriate safe dose range that could be used for subsequent experiments rather than to provide complete toxicity data on the extract. Up to a dose of 3 g/kg both the aqueous and 80% methanolic leaf extracts of C. abyssinica were found to be nontoxic. No mortality was observed in the extract-treated mice and also the extracts did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions and coma.

In vivo hepatoprotective activity studies

In bioassay-directed searching for hepatoprotective agents from natural sources, employing the closely relevant model system to human liver toxicosis could be an effective way to identify therapeutically applicable agents.\textsuperscript{12} CCl\textsubscript{4} is a well-known hepatotoxic chemical commonly used as a chemical inducer of experimental liver injury.\textsuperscript{13} Since the changes associated with CCl\textsubscript{4}-induced liver damage in animal model system are closely related to hepatotoxicity in acute viral hepatitis in human,\textsuperscript{14} CCl\textsubscript{4}-mediated hepatotoxicity was chosen as the experimental model in this study. The main causes of acute liver injury by CCl\textsubscript{4} are free radicals of its metabolites. CCl\textsubscript{4} is reductively bioactivated by cytochrome P450 2E1 into a trichloromethyl radical (CCl\textsubscript{3}), which is subsequently converted into trichloromethyl peroxy radical (OOCCL\textsubscript{3}) in the presence of oxygen. These highly unstable reactive free radical metabolites may cause cellular damage.
damage by initiating lipid peroxidation and covalently binding to macromolecules. They form covalent bonds with unsaturated fatty acids, or take a hydrogen atom from the unsaturated fatty acids of membrane lipids, resulting in the production of chloroform and lipid radicals. The lipid radicals react with molecular oxygen, which initiates peroxidative decomposition of phospholipids in the endoplasmic reticulum. The peroxidation process results in the release of soluble products that may affect cell membrane. Cell membrane integrity is broken and enzymes such as ALT, AST and ALP in cell plasma leak out.[15-17]

Normally, the concentration of transferase in hepatocyte is about 1000-5000 times higher than in serum. When liver is injured by CCl4, membrane permeability of liver parenchyma cell intensified, the activities of ALP, ALT and AST in serum increased sharply as a consequence and serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury in both experimental and clinical setup.[18]

Table 1 shows that CCl4 significantly (P<0.001) increased serum ALP, ALT and AST activities compared with the normal control group. This substantial rise is an indicative of cellular leakage and loss of functional integrity of hepatocytes. However, pretreatment of the rats with the 200 mg/kg of crude extracts, methanol fraction or 100 mg/kg of rutin as well as the standard drug, silymarin inhibited these alterations significantly (P<0.01 to P<0.001).

The aqueous extract reduced serum ALP (65.0%), ALT (46.1%) and AST (58.0%) while, the 80% methanol extract reduced serum ALP (75.4%), ALT (42.3%), and AST (98.0%). The activity of the methanol fraction in preventing CCl4-induced elevation of serum transaminase activities (ALP (85.0%), ALT (75.0%), and AST (79.0%)) was better than those of the aqueous and 80% methanol crude extracts.

Bioassy-guided fractionation of the active methanol fraction of the plant over PTLC followed by sephadex LH-20 purification led to the isolation of the flavonol glycoside rutin. To the best of our knowledge, this is the first report on the isolation of rutin from C. abyssinica. Rutin has been extensively studied and is known to exhibit multiple pharmacological activities including antiviral[19], antitumor[20], antiallergic[21], anti-inflammatory[22], antihypertensive[23], antidiabetic[24], gastroprotective[25], anticonvulsant[26], nephroprotective[27] and antioxidant[28]. In the present study, rutin showed pronounced hepatoprotective activity that was comparable to that of silymarine. Treatment of rats with 100 mg/kg of rutin ameliorated CCl4-induced hepatocellular damage as evidenced by the significant reduction in serum ALP (85.0%), ALT (79.3%) and AST (87.0%) levels. The hepatoprotective activity of rutin was also previously reported. Rutin isolated from Artemisia scoparia Thunb (Asteraceae), a traditional plant used for treatment of liver diseases in Pakistan, has been reported to possess hepatoprotective activity against paracetamol- and CCl4-induced hepato-cellular damage in mice.[29] In another study, administration of rutin to rats pretreated with ethanol has been shown to decrease the levels of liver marker enzymes, lipid peroxidation and significantly elevated the activities of liver superoxide dismutase, catalase, glutathione, glutathione peroxidase, vitamins C and E when compared to untreated ethanol supplemented rats.[30] Furthermore, Radwan et al.[31] reported that rutin prevents radiation induced hepatotoxicity.

Biochemical effects of the crude extracts, fractions and the compound isolated from the leaves of C. abyssinica were supported by the results of histopathological examination, as evidenced by a decrease in the incidence and severity of histopathological hepatic lesions (Figure 1). The histopathological results depicted in Figure 1 demonstrate that liver section of normal control (0.7% CMC treated) rats showing normal hepatic cells with well preserved cytoplasm, prominent nuclei and well brought out central

### Table 1: Effect of the crude extracts, methanol fraction, and rutin isolated from the leaves of *Cineraria abyssinica* in comparison with that of silymarin on activities of serum enzymes in rats injected with 50% CCl4 in paraffin (2 ml/kg) i.p.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>ALP (IU/I)</th>
<th>Serum marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALP (IU/I)</td>
<td>ALT (IU/I)</td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>0.7% CMC</td>
<td>239.6 ± 31.817*</td>
<td>104.2 ± 2.950*</td>
</tr>
<tr>
<td>Toxin</td>
<td>0.7% CMC + CCl4 (2ml/kg)</td>
<td>624.4 ± 209.220</td>
<td>338.6 ± 89.804</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>200 mg/kg + CCl4 (2 ml/kg)</td>
<td>375.2 ± 102.380*</td>
<td>230.6 ± 30.188 (46.1)</td>
</tr>
<tr>
<td>80% Methanol extract</td>
<td>200 mg/kg + CCl4 (2 ml/kg)</td>
<td>334.2 ± 59.757*</td>
<td>239.4 ± 26.435 (42.3)</td>
</tr>
<tr>
<td>Methanol fraction</td>
<td>200 mg/kg + CCl4 (2 ml/kg)</td>
<td>290.0 ± 64.935*</td>
<td>163.6 ± 63.650* (75.0)</td>
</tr>
<tr>
<td>Rutin</td>
<td>100 mg/kg + CCl4 (2 ml/kg)</td>
<td>298.6 ± 81.362*</td>
<td>152.8 ± 108.540* (79.3)</td>
</tr>
<tr>
<td>Silymarin</td>
<td>100 mg/kg + CCl4 (2 ml/kg)</td>
<td>286.8 ± 29.781*</td>
<td>123.2 ± 14.990* (92.0)</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.001) as compared to toxin group and *Significantly different (P<0.01) as compared to toxin group; n = 5, numbers in parenthesis shows % protection.

Compared with the normal group, liver tissue in rats treated with 0.7% CMC revealed extensive liver injuries, characterized by severe hepatocellular degeneration and necrosis (Figure 1B). However, the histopathological hepatic lesions induced by administration of CC14 were remarkably ameliorated by the crude extracts, fractions, rutin or silymarin with different degree. Pretreatment of rats with 200 mg/kg of the aqueous extract showed protection to CC14-induced liver damage as shown by a reduction in liver necrosis (Figure 1C). The small necrosis seen in the aqueous treated group supports the relatively higher transaminase level, compared with the other treated groups observed in the serum markers analysis. Whilst the liver of rats administered with the hydroalcoholic extract showed minor necrosis and focal inflammation (Figure 1D), the liver of those given the methanol fraction had minor focal inflammation without necrosis (Figure 1E). Normal liver histology was observed in rats treated with rutin and silymarin (Figures 1F and 1G). Thus the histological changes associated with the hepatoprotective activity of the crude extracts, methanol fraction, rutin and silymarin strongly support the results of the serum enzymes estimation.

**In vitro radical scavenging activity studies**

Reactive oxygen and nitrogen species (ROS and RNS) contribute to the pathogenesis of various acute and chronic
liver diseases, such as acetaminophen overdose, haemochromatosis, alcoholic liver injury, toxin exposure and viral hepatitis.\textsuperscript{[12-15]} As mentioned earlier, the main cause of hepatic cell death of CCl\textsubscript{4} is focused on their metabolic reactive metabolites to cellular proteins.\textsuperscript{[16,17,36]} As a result, endogenous antioxidants, and covalent binding of the reactive metabolites to cellular proteins.\textsuperscript{[16,17,36]} As a consequence, antioxidants have been proposed as an adjunct therapy for various liver diseases.\textsuperscript{[33]} So in the present study in order to delineate the possible hepatoprotective mechanism of the plant, DPPH free radical scavenging activity was carried out.

In the current study, the crude extracts, fractions, rutin and ascorbic acid were shown to serve as an antioxidant agents or hydrogen donors that can scavenge free radical. As shown in Table 2, rutin showed the highest activity (IC\textsubscript{50} = 3.53 \mu g/ml). Therefore, the hepatoprotective activity of the plant crude extracts, methanol fraction and rutin may be partly attributed to their free radical scavenging activities.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>IC\textsubscript{50} (\mu g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>6.27</td>
</tr>
<tr>
<td>80% Methanol extract</td>
<td>5.78</td>
</tr>
<tr>
<td>Methanol fraction</td>
<td>6.82</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>8.53</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>12.41</td>
</tr>
<tr>
<td>Rutin</td>
<td>3.53</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3.57</td>
</tr>
</tbody>
</table>

**Table 2: DPPH scavenging activity IC\textsubscript{50} values of the crude extracts, solvent fractions, and rutin isolated from the leaves of Cineraria abyssinica in comparison with ascorbic acid**

**CONCLUSION**

The results of the present investigation provide strong evidence that the aqueous and 80\% methanol crude extracts as well as the methanol fraction of *C. abyssinica* significantly inhibit acute liver toxicity induced by CCl\textsubscript{4} in rats, as shown by a reduction of serum liver enzyme activities and the preservation of liver histopathology. The hepatoprotective action of the plant may be attributed to the presence of rutin which showed potent in vitro radical scavenging activity. The results of the study also provide scientific evidence for the traditional use of the plant in the treatment of liver diseases.

**ACKNOWLEDGEMENTS**

The authors are grateful to Mr. Melaku Wondafrash, National Herbarium, Addis Ababa University (AAU) for identification of the plant material and to Dr. Wondeson Ergete and Professor Jakob Schneider, School of Medicine, AAU, for interpreting the histopathology data. One of us (B.S.) is most grateful to the Graduate Studies and Research Office of AAU for sponsoring this study.

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