Flavonoid constituents of Dobera glabra leaves: amelioration impact against CCl₄-induced changes in the genetic materials in male rats

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Introduction

Wild plants are used as sources of food for humans and animals, especially in the areas of insufficient food. Dobera glabra (Forssk.) Poir. (Salvadoraceae) is common in Sudan, Kenya, Uganda, Ethiopia, Saudi Arabia, Yemen and India (Vogt 1995; Aregawi et al. 2008). It is an ever-green tree of up to 8 m with alternate thick skinny leaves. Its flowers are white in color and the fruits are purple, with 1–2 flat seeds (Teklehaymanot & Giday 2010). D. glabra has many common names in folklore medicine according to the region where it is used, among them Garsa (Afarigna), Garas and mikah (Arabic) (Tsegaye et al. 2007).

D. glabra is characterized by its high content of useful minerals and its folkloric use as a tool for forecasting droughts. Fruits of D. glabra are edible and its seeds are essential food at the time of famine (Tsegaye et al. 2007). Its nutritional values are supported by the high content of crude protein, and the edible parts of D. glabra can be compared with that of the common fruits. As a source of human and animal food, in addition to its adaptation to the area, D. glabra is of a great importance for the local people. However, the pastoralists claimed that they could not observe young seedlings of the plant and the available trees of D. glabra are the old ones (Aref et al. 2009). Therefore, a great importance should be given to this plant to avoid its extinction in the near future.

Oxidative stress is the situation of imbalance between the generation and the elimination of reactive oxygen species (ROS) (Khalil et al. 2015), which is characterized by oxidizing biological macromolecules comprising nucleic acid, cellular protein and lipid (Farina et al. 2011). Carbon tetrachloride (CCl₄) is one of the most common mutagens which increase ROS generation.

The principle biological actions of CCl₄ in induction of hepatic damage are due to increasing of the ROS generation, lipid peroxidation and repress the activities of antioxidant enzymes (Kumar et al. 2009). Recently, medicinal plants have gained a remarkable attention, especially those rich in flavonoids and phenolic acids contents, which are known with their antioxidant properties (Grzegorczyk et al. 2007). The harmful effects of genotoxic carcinogens can be overcome by antioxidants’ scavenging of...
ROS and enhancing host antioxidant defense systems (Subapriya et al. 2005).

Despite the importance of *D. glabra* as an edible plant for humans and animals, there are no reports on phytochemical contents and/or biological activity studies regarding to this plant. Therefore, the current study reports the first isolation and identification of bioactive compounds from *D. glabra* leaves with an antioxidant activity and genotoxic protection against CCl₄-induced liver damage in male rats.

### Materials and methods

#### General

1D and 2D NMR experiments (¹H, ¹³C, HMQC and HMBC) were recorded on a Jeol EX-500 spectrometer (JOEL Inc., Tokyo, Japan): 500 MHz (¹H NMR), 125 MHz (¹³C NMR), UV spectrophotometer (Shimadzu UV-240), EI-MS: Thermo scientific ISQ spectrometer (70 eV), ESI mass spectra were measured with a JEOL JMS-HX110 instrument. Column chromatography (CC) Polyamide 6S (Riedel-De-Haen AG, Seelze Hanver, Germany) using MeOH/H₂O as eluent. CC Silica gel 60 (Merck, 0.063–0.2 mm) using CH₂Cl₂:MeOH (2:3). Paper chromatography (PC) (descending) Whatman No. 1 and 3 MM papers, using solvent systems (1) H₂O, (2) 15% HOAc (H₂O:HOAc 85:15), 3) CAW (CHCl₃:HOAc:H₂O 90:45:6), (4) BAW (n-BuOH-HOAc-H₂O 4:1:5, upper layer), (5) (C₆H₅)n-BuOH:H₂O:pyridine 1:5:3, upper layer). Solvents 4 and 5 were used for sugar analysis, Sephadex LH-20 (Pharmazia). Authentic samples were obtained from the department of phytochemistry and plant systematics, NRC. Complete acid hydrolysis for O-glycosides (2 N HCl, 2 h, 100 °C) was carried out and followed by paper co-chromatography with authentic samples to identify the aglycones and sugar moieties.

#### Plant material

Fresh sample of *D. glabra* was collected from Sudan in April 2011. The sample was identified by Prof. Dr S.A. Kawashy. A voucher specimen (No. M89) has been deposited in the herbarium of the National Research Center, Dokki, Giza, Egypt (CAIRC).

#### Extraction and isolation

Air-dried, ground, leaves of *D. glabra* (1700 g) were extracted four times at room temperature with 70% methanol/water. The aqeous methanol extract was evaporated under reduced pressure and temperature to obtain a residue of 280 g (16.47%) of *D. glabra* extract (DGE). The DGE was applied to polyamide CC (125 × 6.0 cm²), eluting with MeOH/H₂O mixtures of decreasing polarities to yield four main fractions. Each of them was eluted separately on preparative paper chromatography (PPC) using H₂O, 15% HOAc and BAW, as eluents to afforded compounds 1–7. Final purification was achieved by a combination of PPC and repeated Sephadex LH-20 column.

#### Drugs and reagents

Carbon tetrachloride (CCl₄, 99.9% purity) was purchased from Sigma (St. Louis, MO). Trizol was bought from Invitrogen (Carlsbad, CA). The reverse transcription and PCR kits were obtained from Fermentas (Glen Burnie, MD). SYBR Green Mix was purchased from Stratagene (La Jolla, CA).

### Experimental animals

Eighty adult albino male rats (100–120 g, purchased from the Animal House Colony, Giza, Egypt) were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, National Research Center, Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were divided into groups (10 rats/group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23 ± 1 °C) and artificially illuminated (12 h dark/light cycle) room, free from any source of chemical contamination. All animals received care in compliance with the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

### Treatments of animals with DGE

The male rats were randomly allocated in 8 groups (10 rats/group) and treated for 8 weeks as follows: Group 1, control group: animals were treated intragastrically with solvent vehicle control (olive oil); Group 2, animals were injected intraperitoneally with 0.5 mL/kg of CCl₄ (suspended in olive oil, 1:9 v/v) twice weekly to induce hepatic damage; Groups 3–5, animals were treated intragastrically with 50, 100 and 200 mg/kg bw of DGE; Groups 6–8, animals were treated with CCl₄ as in groups 2 and then with DGE (50, 100 and 200 mg/kg bw), respectively.

#### Tissue collection

At 24 h after the last injection, rats in each group were sacrificed by decapitation after anesthetized. The liver tissues were collected on ice bath and then used to investigate apoptosis, ROS, isolate total RNA (for the determination of expression (mRNA) of apoptotic related genes GLAST, GLT-1, SNAT3), isolate DNA (for the determination DNA fragmentation) and GPx activity was measured.

#### Determination of ROS formation

Intracellular ROS generation was measured in liver tissues by flow cytometry with an oxidation-sensitive DCFH-DA fluorescent probe, after single-cell suspensions were made. DCFH-DA is a non-fluorescent compound that is freely taken up into cells. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) by intracellular ROS and enhancing host antioxidant defense systems (Subapriya et al. 2005).

#### Gene expression analysis

### Extraction of total RNA and cDNA synthesis

Liver tissues of male rats were used to extract the total RNA using TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) Kit, according to the manufacturer’s instructions. Approximately
50 mg of the liver tissues were mixed with some drops of liquid nitrogen and homogenized in 1 mL of TRIZol® Reagent in autoclaved mortar. Afterwards, total RNA was dissolved and preserved in diethylpyrocarbonate (DEPC)-treated water.

To assess the RNA yield and purity of the total RNA, RNase-free DNase I (Invitrogen, Germany) was used to digest DNA contamination. A small drop of isolated RNA was examined photospectrometrically at 260 nm. The purity of total RNA was considered between ration of 1.8 and 2.1 to be of good purity when examined by photospectrometer at the 260/280 nm ratio. To avoid RNA degradation, aliquots of prepared RNA after isolation either reverses transcribed or stored at −80°C until to use.

To synthesize the complementary DNA (cDNA) isolated RNA from liver, tissues were reverse transcribed. The reaction volume was carried out in 20 μL. The reaction was prepared according to the instructions of the RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The reverse transcription (RT) reaction was performed for 10 min at 25°C. Afterwards, the tubes of the reaction were put in thermo-cycler machine for 60 min at 42°C, and then the reaction was terminated for 5 min at 99°C. The PCR products containing the cDNA were kept at −20°C until to use for cDNA amplification.

Quantitative real-time-PCR (qRT-PCR)

A StepOne Real-Time PCR System (Applied Biosystem, Foster City) was used to assess the copy of the cDNA of male rats to detect the expression values of the tested genes. To perform the PCR reaction, the instructions of the RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The reverse transcription (RT) reaction was performed for 10 min at 25°C. Afterwards, the tubes of the reaction were put in thermo-cycler machine for 60 min at 42°C, and then the reaction was terminated for 5 min at 99°C. The PCR products containing the cDNA were kept at −20°C until to use for cDNA amplification.

DNA fragment analysis

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described according to Lu et al. (2002). Briefly, liver tissues were homogenized, washed in PBS, and lysed in 0.5 mL of DNA extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton, and 100 μg/mL proteinase K, pH 8.0) for overnight at 37°C. The lysate was then incubated with 100 μg/mL DNase free RNase for 2 h at 37°C, before three extractions with an equal volume of phenol/chloroform (1:1 v/v), and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4°C. The extracted DNA was precipitated in two volume of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at −20°C for 1 h, followed by centrifuging at 15,000 rpm for 15 min at 4°C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris- HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris-acetate/EDTA buffer (TAE) (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet trans-illumination.

Determination of glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured by a procedure according to Miranda et al. (1995). The reaction mixture consisted of 8 mM H₂O₂, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5, and a suitable amount of the enzyme preparation from the liver tissue homogenate. The change in absorbance at 470 nm due to guaiacol oxidation was followed at 30 s intervals. One unit of GPx activity was defined as the amount of enzyme which increases the O.D. by 1.0/min under standard assay conditions.

Statistical analysis

All results were expressed as mean ± SE of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) program, version 11 followed by least significant difference (LSD) to compare significance between groups. Difference was considered significant when p > 0.05.

Results and discussion

Identification of flavonoid compounds

The aqueous methanol extract of the aerial parts of D. glabra (DGE) was purified through chromatographic methods yielding seven flavonoid compounds (Figure 1). Their structures were elucidated by chemical and spectroscopic techniques (Mabry et al. 1970; Agrawal & Bansal 1989; Markham & Geiger 1994), and identified as isorhamnetin-3-O-β-glucopyranoside-7-O-α-rhamnopyranoside (1), isorhamnetin-3-O-α-rhamnopyranoside-7-O-β-glucopyranoside (2), kaempferol-3,7-di-O-α-rhamnopyranoside (3), isorhamnetin-3-O-β-glucopyranoside (4), kaempferol-3-O-β-glucopyranoside (5), isorhamnetin (6) and kaempferol (7). Spectral data of the isolated flavonoids were consistent with those previously published (Beninger et al. 1998; Hussein et al. 2009, 2011; Yi et al. 2011).

The antioxidant properties of flavonoids make their existence in low amounts in foods or body, compared with that of an oxidizable substance, as they inhibit the oxidation of that substance. Therefore, the present study aimed to investigate the potential...
Antioxidant activity of *D. glabra* extract against CCl₄ induced oxidative stress in male rats.

**Antioxidant activity of *D. glabra* leaves extract (DGE) against CCl₄ induced oxidative stress**

In the current study, the effect of DGE on ROS generation, alteration of expression of GLAST, GLT-1 and SNAT antioxidant-related genes, DNA fragmentation and the antioxidant enzyme GPx levels against CCl₄ inducing oxidative stress in male rats were investigated.

The effect of CCl₄ on intracellular ROS changes and the suppression effect of DGE in liver tissues of male rats are summarized in Figure 2. CCl₄ increased significantly the intracellular ROS levels by 523.1% compared to the control group. The results revealed that DGE reduced significantly the production of intracellular ROS induced by CCl₄ compared to CCl₄ alone, especially at DGEH (200 mg/kg bw). However, there were no significant differences of ROS between control and different doses of DGE.

The expression of antioxidant related genes (GLAST, GLT-1 and SNAT) was quantified by real-time RT-PCR. In DGE supplemented animals GLAST, GLT-1 and SNAT3 expressions elevated similar expression values to control group (Figure 3). However, CCl₄ exposure decreased the mRNA expression values of GLAST, GLT-1 and SNAT3 to 36.6%, 40.6% and 39.1% of control, respectively (Figure 3). In contrast, comparing with CCl₄ alone, expression values of GLAST gene increased 1.33, 1.98 and 2.16-fold in DGE L + CCl₄, DGE M + CCl₄ and DGE H + CCl₄, respectively (Figure 3). In the same line, expression values of GLT-1 gene increased 1.53-, 1.66- and 1.72-fold in DGE L + CCl₄, DGE M + CCl₄ and DGE H + CCl₄, respectively (Figure 3). In addition, expression values of SNAT3 mRNA increased to 1.87-, 2.02-, and 2.09-fold in DGE L + CCl₄, DGE M + CCl₄ and DGE H + CCl₄, respectively (Figure 3).

Determination of rate of DNA fragmentation in liver tissues of male rats following CCl₄ and/or *Dobra* extract as an alternate for oxidative stress-induced DNA damage is shown in Figure 4. The results showed that the DNA fragmentation rates following different doses of *Dobra* extracts treatment were relatively similar to that of the control. However, the DNA fragmentation increased significantly following CCl₄ treatment compared to that of the control (Figure 4). In contrary, the rate of DNA fragmentation decreased significantly following CCl₄ plus low, medium or high dose of DGE compared with CCl₄ alone (Figure 4).

Table 2 shows the suppression effect of DGE on CCl₄-induced alteration in the antioxidant enzyme GPxs. Comparing with the control group, GPx activity levels were relatively similar with those in DGE treatments (50, 100 and 200 mg/kg bw). However, the activity level of GPxs decreased significantly in CCl₄ treatment compared with the control group. On the other hand, comparing
with the CCl₄ group alone GPx (1.8 U/mg protein/min), GPx concentrations increased significantly in DGE L+CCl₄, DGE M+CCl₄ and DGE H+CCl₄ groups to 2.7, 3.4 and 4.8 U/mg protein/min, respectively (Table 2). Data in the present study revealed that CCl₄ induced genetic toxicity in the form of alterations in gene expression and DNA damage as compared to control rats. These finding are in agreement with that reported by Abdou et al. (2012). The DNA damage can originate from its direct modification by chemical agents or their metabolites; from the processes of DNA excision repair, replication and recombination; or from the process of apoptosis (Eastman & Barry 1992). If left un-repaired, oxidative DNA damage can lead to detrimental biological consequences in organisms, including mutations and transformation of cells to malignant cells and cell death. Previous studies showed that changes in genomic DNA could reflect DNA alterations from single base changes (point mutations) to complex chromosomal rearrangements (Atienzar et al. 1999, 2002).

The action mechanism of which CCl₄ induced toxicity is following CCl₄ administration, it is activated by cytochrome P₄₅₀.
system to form trichloromethyl (CCl₃) radical. The reaction between trichloromethyl (CCl₃) radical and DNA is thought to function as initiator of liver pathogenesis including cirrhosis, genotoxicity of hepatic tissue and hepatic cancer.

Our data revealed that co-administration of DGE reduced significantly the ROS generation and modulate the activity levels of the antioxidant enzyme GPx in male rats with hepatic injury induced by CCl₄. Within the excitatory amino acid transporters (EAATs), the glutamate transporters, GLAST, GLT-1 and SNAT3 are the main players in provoking glutamate uptake into astrocytes in the CNS. Therefore, it was hypothesized that GLAST, GLT-1 and SNAT3 played an important role in maintaining GSH in the CNS (Yin et al. 2007). In the current work, we found that the mRNA expression of the glutamate transporters was down-regulated obviously in CCl₄ alone. DGE supplementation, especially at 200 mg/kg bw (DGE H) counteracted the CCl₄ effect on GLAST, GLT-1 and SNAT3 mRNA expressions. To date, there are few in vivo studies focusing on this point.

Therefore, it could be assumed that D. glabra extract was able to inhibit the induction of oxidative stress by CCl₄ in male rats. To our knowledge, this study is the first one to investigate the protective effect of D. glabra against CCl₄-induced genotoxicity in male rats.

There is an increasing evidence that a plethora of plant components including minerals and flavonoids can act as inhibitors of toxicity (Mitić-Culafić et al. 2005). However, Guinand and Lemessa (2001) reported that the total mineral content of D. glabra leaves (including Ca, P, Mg and Na.) is relatively higher than most of the 540 browse plant species of West and East Africa. It contributes 17.37–21.17% of the dry matter. It is known that chemoprotective agents are capable of exerting their antigenotoxic effects by one mechanism or a combination of mechanisms. These mechanisms include the scavenging of ROS, the reducing of formation of reactive carcinogenic-metabolites, the inducing of carcinogen-detoxifying enzymes, the effect on apoptosis and the inhibition of cell proliferation (Premkumar et al. 2004; Chandrasekar et al. 2006). Meanwhile, gene transcription is directly influenced by antioxidants through the presence of antioxidant response elements in the promoters of many genes (Palmer & Paulson 1997). In consistent with these findings, our results indicated that the pharmacological properties of D. glabra extract, which are mostly described as antioxidants, might explain the way in which it protects the liver tissues against ROS generation, DNA fragmentation and alteration in the expression of GLAST, GLT-1 and SNAT3 as well as GPx activity induced by CCl₄.

**Conclusions**

This is the first report of the isolation of flavonoid compounds from D. glabra and its genotoxic activity evaluation. The results of the study demonstrate that administration of D. glabra may be valuable in the treatment and amelioration of hepatic oxidative stress and genotoxicity.

**Disclosure statement**

The authors report no declarations of interest.

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**References**

Abdou HS, Salah SH, Booles HF, Abdel Rahim EA. 2012. Antioxidant effect of celery against carbontetrachloride induced hepatic damage in rats. Afr J Microbiol Res 6:5657–5667.
Agrawal PK, Bansal MC. 1989. Flavonoid glycosides. In: Agrawal PK, Editor. Carbon-13 NMR of flavonoids. New York: Elsevier, p. 283–364.

Aref IM, El Atta HA, Al Ghtani AA. 2009. Ecological study on *Debera glabra* Forsk. At Janaz in Saudi Arabia. J Hortic For. 1:198–204.

Aregawi T, Melaku S, Nigatu L. 2008. Management and utilization of browse species as livestock feed in semi-arid district of North Ethiopia. Livest Res Rural Dev 20. [cited 2016 June 11]. Available from: http://www.lrrd.org/lrrd20/6/areg20086.htm

Atienzar FA, Conradi M, Evenden AJ, Jha AN, Depledge MH. 1999. *Aregawi T, Melaku S, Nigatu L. 2008. Management and utilization of browse species as livestock feed in semi-arid district of North Ethiopia. Livest Res Rural Dev 20. [cited 2016 June 11]. Available from: http://www.lrrd.org/lrrd20/6/areg20086.htm

Atienza FA, Venier P, Jha AN, Depledge MH. 2002. Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. Mutat Res/Genet Toxicol Environ Mutagen. 521:151–163.

Beninger CW, Hosfield GL, Nair MG. 1998. Flavonol glycosides from the seed coat of a new mangeta-type dry bean (*Phaseolus vulgaris* L.). J Agric Food Chem. 46:2906–2910.

Chandrasekar MJN, Bommu P, Nanjan MJ, Suresh B. 2009. Chemical study on Flavonoids of *Nitraria retusa* L. (Zygophyllaceae). Biochem Syst Ecol. 39:778–780.

Kassem MES, Ibrahim LF, Hussein SR, El-Sharawy R, El-Ansari MA, Hassaname MM, Booles HF. 2016. Myricitrin and bioactive extract of *Albizia amara* leaves: DNA protection and modulation of fertility and antioxidant-related genes expression. Pharm Biol., in press doi:10.3109/13880209.2016.1158285.

Khalil HS, Goltsov A, Langdon SP, Harrison DJ, Bown J, Deeni Y. 2015. Comparative study on the antibacterial activity of volatiles from *Salvia officinalis* L., *Arch Biol Sc.* 57:173–178.

Premkumar K, Abraham SK, Santhiya ST, Ramesh A. 2004. Protective effect of spirulina fusiformis on chemical-induced genotoxicity in mice. Fittoterapia. 75:24–31.

Subapriya R, Kumaraguruparan R, Abraham SK, Nagini S. 2005. Protective effects of ethanolic neem leaf extract on *N*-methyl-`N`-nitro-`N`-nitrosoguanidine-induced genotoxicity and oxidative stress in mice. Drug Chem Toxic. 27:15–26.

Toklehyamton T, Giday M. 2010. Ethnobotanical study of wild edible plants of Kara and Kwego semi-pastoralist people in Lower Omo River Valley, Debub Omo Zone, SNNPR, Ethiopia. J Ethnobiol Ethnomed. 6:23.

Vogt K. 1995. A field worker’s guide to the identification, propagation and uses of common trees and shrubs of dryland Sudan. UK: SOS Sahel International. p. 167.

Yi Y, Wu X, Wang Y, Ye WC, Zhang QW, 2011. Studies on the flavonoids from the flowers of *Hylocereus undatus*. Zhong Yao Cai. 34:712–716.