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Hepatoprotective Effect of Flavonol Glycosides Rich Fraction from Egyptian *Vicia calcarata* Desf. Against CCl_4-Induced Liver Damage in Rats

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The hepatoprotective activity of flavonol glycosides rich fraction (F-2), prepared from 70% alcohol extract of the aerial parts of *V. calcarata* Desf., was evaluated in a rat model with a liver injury induced by daily oral administration of CCl_4 (100 mg/kg, b.w) for four weeks. Treatment of the animals with F-2 using a dose of (25 mg/kg, b.w) during the induction of hepatic damage by CCl_4 significantly reduced the indices of liver injuries. The hepatoprotective effects of F-2 significantly reduced the elevated levels of the following serum enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The antioxidant activity of F-2 markedly ameliorated the antioxidant parameters including glutathione (GSH) content, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), plasma catalase (CAT) and packed erythrocytes glucose-6-phosphate dehydrogenase (G6PDH) to be comparable with normal control levels. In addition, it normalized liver malondialdehyde (MDA) levels and creatinine concentration. Chromatographic purification of F-2 resulted in the isolation of two flavonol glycosides that rarely occur in the plant kingdom, identified as quercetin-3, 5-*di*-*O*-β-D-diglucoside (**5**) and kaempferol-3, 5-*di*-*O*-β-D-diglucoside (**4**) in addition to the three known compounds identified as quercetin-3-*O*-α-L-rhamnosyl- (1→6)-β-D-glucoside [rutin, **3**], quercetin-3-*O*-β-D-glucoside [isoquercitrin, **2**] and kaempferol-3-*O*-β-D-glucoside [astragalin, **1**]. These compounds were identified based on interpretation of their physical, chemical, and spectral data. Moreover, the spectrophotometric estimation of the flavonoids content revealed that the aerial parts of the plant contain an appreciable amount of flavonoids (0.89%) calculated as rutin. The data obtained from this study revealed that the flavonol glycosides of F-2 protect the rat liver from hepatic damage induced by CCl_4 through inhibition of lipid peroxidation caused by CCl_4 reactive free radicals.

Key words: *Vicia calcarata* Desf., Aerial parts, Fabaceae, Flavonol glycosides, Hepatoprotective effect, Antioxidant effect

INTRODUCTION

It is well known that a substantial increase in steatosis and fibrosis usually leads to potentially lethal cirrhosis of the liver in humans (Ulicna *et al.*, 2003). The high global prevalence of these hepatopathies places them among the most serious diseases. Although the pathogenesis of liver fibrosis is not entirely clear, there is no doubt that reactive oxygen species (ROS) play an important role in pathological changes in the liver, particularly in cases of alcoholic and toxic liver diseases (Poli and Parola, 1997). Several endogenous protective mechanisms have evolved to limit ROS and the damage caused by them (Sies, 1993). However, since this may not offer complete protection, or when the formation of ROS is excessive, additional protective mechanisms of dietary antioxidants may be of great importance. Therefore, many natural antioxidants have been proposed to prevent and treat hepatopathies induced by oxidative stress (Lieber, 1997;

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Cervinkova & Drahota, 1998). There is increasing evidence for the hepatoprotective role of hydroxy- and polyhydroxy organic compounds- particularly from vegetables, fruits and herbs (Bass, 1999). *Vicia calcarata* Desf. or *Vicia monantha* Retz is indigenous to the Mediterranean region (Bedivian, 1936). It is commonly known in Arabic as Duhhrag, Udays, and Kharig, being very common in the Nile valley particularly distributed in clover and wheat fields and it is used as an animal feeder (Tackholm, 1956). The aerial parts of the entitled plant contain an appreciable amount (0.89%) of flavonoids. Therefore, it may be anticipated that it would have health promoting effects in humans. This paper deals with the potential hepatoprotective effect of F-2, a rich fraction with kaempferol and quercetin glycosides, against CCl_4-induced hepatic damage in rats as well as isolation and identification of its flavonoids content.

MATERIALS AND METHODS

Phytochemical study

Plant materials

Aerial parts of *Vicia calcarata* Desf. were collected prior to the flowering stage (February 2002) from the Wadi Al-Arish area, North Sinai, Egypt. Professor Abd El Salaam M. Al-Nowiahi, Professor of Plant Taxonomy, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt kindly identified the plant materials. A voucher specimen (VC-2002) has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University.

Apparatus and chemicals

The UV spectra were measured using a Hitachi U–3200 spectrophotometer. The UV spectral data of the isolated compounds were recorded following standard procedures (Mabry *et al.*, 1970). ^{1}H-, ^{13}C-NMR, and 2D-NMR spectra were recorded using a JEOL JNM-LA-500 spectrophotometer in DMSO-d_6. Chemical shifts were given in δ values (ppm) with TMS as the internal standard. HRFAB-MS and FAB- MS data were registered in negative and positive ion mode using a JEOL JMS-DX 302 spectrophotometer. TLC was performed on silica gel 60 F_{254} sheets, 0.25 mm thickness, RP-C_{18} TLC, 10×20 cm, and pre-coated cellulose plates, without fluorescent indicator, 20×20 cm, 0.5 mm thickness purchased from E-Merck Darmstadt, Germany. Ion exchange resin, Diaion HP-20 (Nippon, Rensui CO, Japan) and Sephadex LH-20, 25-100 μm (Pharmacia Inc, Sweden) were used for CC fractionation and purification. The Knaüer HPLC series (Berlin, Germany) equipped with maxi-star K-1001 and mini-star K-501 pumps, Knaüer dynamic mixing chamber, Knaüer EI. Driven K-6 injector with 20 μL loop, Knaüer UV detector 2501, and reversed phase C-18 column, 250×21.20 mm, with particle size of 10 μm (Luna, Phenomenex, U.S.A.) was used to purify the compounds **4** and **5**.

Determination of flavonoids content

The total flavonoids of the aerial parts of *V. calcarata* Desf. were estimated following the method described by Singab (2002). The total flavonoids content (0.89%) was calculated using the pre-established standard calibration curve using different concentrations of rutin. A linear relationship obeying Beer's law was obtained for the range of rutin concentrations used (20-240 μg/10 mL).

Plant extraction and fractionation

One kg of the air-dried aerial parts of *V. calcarata* Desf. was powdered and extracted with 70% alcohol at room temperature until exhaustion. The extract was concentrated under reduced pressure at temperature not exceeding 50°C in order to give 150 g of semi-solid residue. 70 g of the semi-solid extract were dissolved in a small amount of distilled water and were fractionated over Diaion HP–20 CC eluted with H_2O (F-1), 50% MeOH (F-2), and finally with methanol (F-3). Each fraction was concentrated under reduced pressure at temperature not exceeding 50°C in order to give 25 g, 35 g, and 9 g of the fractions respectively.

TLC analysis of the three fractions, F-1, F-2 and F-3, indicated that F-1 did not contain any flavonoid compounds, while F-2 displayed several spots of flavonoid glycosides with different fluorescence under a UV lamp at 365 nm. Only two spots of the flavonoid aglycones in F-3 could be identified by Co-TLC being authentic as quercetin and kaempferol. Ed. Note: confirm wording.

Isolation of flavonol glycosides (1~ 5)

20 g of F-2 were chromatographed in portions over LH-20 CC eluted with 70% methanol. Similar fractions were pooled together resulting in 10 main fractions. Fractions 8 and 9 showed two major spots on TLC (pre-coated cellulose) developed with *n*-butanol: acetic acid: water-4:1:5. Thus, the two fractions were collected and subjected to preparative cellulose TLC using the solvent system mentioned earlier. Each band was eluted separately with methanol and concentrated under reduced pressure at temperature not exceeding 50°C. Purification of each material over small LH-20 CC eluted with MeOH produced 10 mg of yellow powder of compound **1** and 50 mg of yellow needle crystals of compound **2**. Fraction 6 displayed a yellow deposit and produced 55 mg of yellow powder of compound **3** on recrystallization from hot methanol. Fractions 3, 4, and 5 displayed two spots with blue and canary yellow fluorescence under a UV lamp at 365 nm using reversed phase TLC (RP-18) developed with MeOH:H_2O-1:1. These fractions were collected and subjected to prep-HPLC using MeOH:H_2O-1:1 as a mobile

phase, isocratic elution, a UV detector at 365 nm with flow rate 5 mL/min and prep. C-18 column to produce two major peaks with R_t 14 and 15 min respectively. Each material was collected and concentrated then purified over LH-20 CC eluted with 90% MeOH followed by recrystallization from hot methanol to produce 8 mg of yellowish white powder of compound **4** and 100 mg of golden yellow crystals of compound **5**.

Acid hydrolysis of 1~5

Each compound (2 mg) was refluxed in 10 mL of 0.1N H_2SO_4 for four hours. The aglycones were extracted with diethyl ether and identified by comparison with authentic samples on silica gel TLC (Petrol: acetone -9:1 & Petrol: EtOAc- 95:5). The aq. layer was neutralized with $NaHCO_3$ and freeze-dried. The sugar was extracted with pyridine and the solvent was evaporated. The residue dissolved in a small amount of MeOH and run on silica gel TLC in EtOAc: MeOH: H_2O: HOAc -57:13:13:17 using ρ-anisidine phthalate as the detection reagent.

Kaempferol-3-*O*-β-D-glucopyranoside (astragalin, 1)

Kaempferol-3-*O*-β-D-glucopyranoside (astragalin, 1) was obtained as a yellow powder (10 mg) with R_f 0.65 using silica gel TLC ($CHCl_3$: MeOH-7.5: 2.5). FAB-MS (-ve) m/z: 447, 285. ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm), δ 7.97 (2 H, *d, J* = 8.8 Hz, H-2', H-6`), δ 6.84 (2 H, *d, J* = 8.8 Hz, H-3`, H-5`), δ 6.11 (1H, *s*, H-8), δ 6.0 (1H, *s*, H-6), δ 5.32 (1H, *d, J* = 7.3 Hz, H-1'). ^{13}C–NMR (125 MHz, DMSO-d_6, δ ppm), 175.4 (C-4), 163.2 (C-7), 160.8 (C-5), 160.7 (C-4'), 157.1 (C-9), 154.2 (C-2), 135.7 (C-3), 130.7(C-2' & C-6'), 120.9 (C-1'), 115.0 (C3'-C5'), 104.9 (C-10), 101.8 (C-1"), 99.8 (C-6"), 94.8 (C-8), 77.2 (C-3"), 76.5 (C-5"), 74.2 (C-2"), 69.5 (C-4"), 60.8 (C-6").

Quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin, 2)

Quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin, 2) was obtained as yellow needle crystals (50 mg) with R_f 0.63 using silica gel TLC ($CHCl_3$:MeOH-7.5:2.5). FAB-MS (+ve) m/z: 465, 303. ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm), δ 7.58 (1H, *d, J* = 2.4 Hz, H-2'), δ 7.56 (1H, *dd, J* = 2.4 & 8.6 Hz, H-6'), δ 6.84 (1H, *d, J* = 8.6 Hz, H-5'), δ 6.39 (1H, *d, J* = 2.0 Hz, H-8), δ 6.19 (1H, *d, J* = 2.0 Hz, H-6), δ 5.45 (1H, *d, J* = 7.3 Hz, H-1"). ^{13}C-NMR (125 MHz, DMSO-d_6, δ ppm), 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-9), 156.1 (C-2), 148.4 (C-4'), 144.7 (C-3'), 133.3 (C-3), 121.5 (C-1'), 121.1 (C-6'), 116.1 (C5'), 115.2 (C-2'), 103.9 (C-10), 100.8 (C-1"), 98.6 (C-6), 93.5 (C-8), 77.5 (C-3"), 76.5 (C-5"), 74.0 (C-2"), 69.9 (C-4"), 60.9 (C-6").

Quercetin-3-*O*-α-L-rhamnopyanosyl-(1→6)-β-D-glucopyranoside (rutin, 3)

Quercetin-3-*O*-α-L-rhamnopyanosyl-(16)-β-D-glucopyranoside (rutin, 3) was obtained as a yellow powder (55 mg) with R_f 0.38 using silica gel TLC ($CHCl_3$: MeOH-7:3). FAB-MS (+ve) m/z: 611, 465, 303. ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm), δ 7.55 (1H, *d, J* = 2 Hz, H-2'), δ 7.50 (1H, *dd, J* = 2.4 & 8.4 Hz, H-6'), δ 6.82 (1H, *d, J* = 8.4 Hz, H-5'), δ 6.35 (1H, *d, J* = 2.2 Hz, H-8), δ 6.11 (1H, *d, J* = 2.2 Hz, H-6), δ 5.02 (1H, *d, J* = 7.4 Hz, H-1"), δ 4.48 (1H, *d, J* = 1.2 Hz, H-1'"), δ 1.2 (3H, *d, J* = 6 Hz, Rha-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6, δ ppm), 177.5 (C-4), 163.6 (C-7), 162.2 (C-5), 159.3 (C-9), 157.1 (C-2), 148.6 (C-4'), 144.4 (C-3'), 135.5 (C-3), 122.1 (C-1'), 121.7 (C-6'), 116.5 (C-5'), 115.1 (C-2'), 104.3 (C-10), 103.8 (C-1"), 102.0 (C-1'"), 99.6 (C-6), 93.5 (C-8), 78.0 (C-5"), 77.5 (C-3"), 74.9 (C-2"), 72.8 (C-4"), 71.9 (C-3'"), 71.7 (C-2'"), 71.1 (C-4'"), 69.1 (C-5'"), 66.5 (C-6"), 17.3 (C-6'").

Kaempferol-3, 5-*di*-*O*-β-D-glucopyranoside (4)

Kaempferol-3, 5-*di*-*O*-β-D-glucopyranoside (**4**) was obtained as a yellowish white powder (8 mg). It gave an intense blue fluorescent spot under a UV lamp at 365 nm with R_f 0.58 using RP-C_{18} silica gel TLC (MeOH: H_2O-1: 1). FAB-MS (-ve) m/z: 609, 447, 285. UV λ_{max}^{MeOH} nm (log ε): 260 (4.20), 345 (4.28); (+NaOMe) 270,322 sh, 394; (+$AlCl_3$) 268, 417; (+$AlCl_3$/HCl) 268, 400; (+NaOAc) 270, 375. ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm), δ 7.95 (2H, *d, J* = 8.8 Hz, H-2', H-6'), δ 6.80 (2H, *d, J* = 8.8 Hz, H-3', H-5'), δ 6.60 (1H, *s*, H-8), δ 6.42 (1H, *s*, H-6), δ 5.20 (1H, *d, J* = 7.95 Hz, H-1"), δ 4.64 (1H, *d, J* = 7.32 Hz, H-1'"). ^{13}C–NMR (125 MHz, DMSO-d_6 -δ ppm), 172.8 (C-4), 162.6 (C-7), 159.6 (C-5), 158.7 (C-4'), 157.7 (C-9), 153.6 (C-2), 135.1 (C-3), 130.9 (C-2`&C-6`), 120.9 (C-1'), 114.9 (C3'-C5`), 106.0 (C-10), 104.3 (C-6), 101.4 (C-1"), 101.3 (C-1'"), 97.8 (C-8), 77.5 (C-5"), 77.3 (C-5'"), 76.6 (C-3"), 76.5 (C-3'"), 74.2 (C-2"), 73.6 (C-2'"), 70.4 (C-4"), 69.7 (C-4'"), 60.8 (C-6"), 60.7 (C-6'").

Quercetin-3, 5-*di*-*O*-β-D-glucopyranoside (5)

Quercetin-3, 5-*di*-*O*-β-D-glucopyranoside (**5**) was obtained as golden yellow crystals (100 mg). It gave an intense canary yellow fluorescent spot under a UV lamp at 365 nm with R_f 0.55 using RP-C_{18} silica gel TLC (MeOH: H_2O-1:1). HRFAB-MS (-ve) m/z: 625.1410 consistent with $C_{27}H_{29}O_{17}$ (calc.625.1405). FAB-MS (-ve) m/z, 625, 463, and 301. UV λ_{max}^{MeOH} nm (log ε): 265 (4.0), 350 (4.4); (+NaOMe) 252, 323sh, 351; (+$AlCl_3$) 254, 303, 356; (+$AlCl_3$/HCl) 252, 305, 351; (+NaOAc) 270, 315, 364, 375, 387. ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm), δ 7.57 (1H, *d, J* = 2.4 Hz, H-2'), δ 7.56 (1H, *dd, J* = 2.4 & 8.3 Hz, H-6'), δ 6.83 (1H, *d, J* = 8.3 Hz, H-5'), δ 6.73 (1H, *d, J* = 1.8 Hz, H-8), δ 6.49 (1H, *d, J* = 1.8 Hz, H-6), δ 5.28 (1H, *d, J* = 7.3 Hz, H-1"), δ 4.71 (1H, *d, J* = 7.6 Hz, H-1'"). ^{13}C-NMR (125 MHz, DMSO-d_6, δ ppm), 172.9 (C-4), 162.3 (C-7), 158.7 (C-5), 157.6 (C-9), 157.1 (C-2), 148.4 (C-4'), 144.4 (C-3'),

135.3 (C-3), 121.3 (C-1'), 121.0 (C-6'), 116.0 (C-5'), 115.1 (C-2'), 106.5 (C-10), 104.7 (C-6), 104.4 (C-1''), 101.3 (C-1'''), 97.6 (C-8), 77.5 (C-5''), 77.3 (C-5'''), 76.6 (C-3''), 76.5 (C-3''), 74.1 (C-2''), 73.6 (C-2'''), 69.8 (C-4''), 69.6 (C-4'''), 61.0 (C-6''), 60.7 (C-6''').

Biological study

Chemicals used for biological study

All chemicals and biochemical kits were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carbon tetrachloride was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Animals

Male Swiss albino rats (120-150 mg) were obtained from the Egyptian Organization for Biological Product and Vaccines, Giza, Egypt. Animals were housed in cages with good ventilation and illumination. Animals had access to unlimited water and standard rodent chow. Animal maintenance and treatments were conducted in accordance with the National Institute of Health Guide for Animal, as approved by the Institutional Animal Care and Use Committee (IACUC).

Lethal dose determination (LD_{50})

The adult male Swiss albino rats were subjected to a series of different concentrations of F-2. The concentrations (15-120 mg/kg, b.w) were dissolved in 0.1 mL of DMSO then completed to suitable volume with sterile saline. The concentrations were administrated orally to six rats for each concentration. Probability analysis following the method described by Hardison *et al.* (1983) was used to calculate the lethal dose on rats and the recorded mortality was detected within 40 days post-treatment. Therefore, a non-toxic dose (25 mg/kg, b.w) was chosen for the subsequent study.

Experimental procedures

The animals were divided into four groups with six rats in each group. Group 1 was the control group and received a combination of 0.25 mL of olive oil and 0.1 mL DMSO diluted with saline up to 1 mL. Group 2 received daily oral doses of CCl_4 (100 mg/kg, b.w) for four weeks. Group 3 received daily oral doses of F-2 (25 mg/kg, b.w) for four weeks. Group 4 received daily oral doses of a combination of F-2 (25 mg/kg, b.w) and CCl_4 (100 mg/kg, b.w) for four weeks. The last three groups of animals received the calculated doses dispersed in the same liquid combination as was used in control group.

Measurement of biochemical parameters

Samples collection

Samples were collected after one month from the beginning of the experiments. The animals were fasted overnight prior to collection of each sample. The whole blood was collected by heart puncture after a light anesthesia using heparinized syringes. One part was used for glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) estimations. The separated plasma from the heparinized blood was used for the determination of catalase (CAT) activity and malondialdehyde (MDA) concentration. Packed erythrocytes were used to measure glucose-6-phosphate dehydrogenase (G6PDH) activity. Liver function parameters including, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) as well as creatinine concentration were estimated from the plasma.

Analytical procedures

Lipid peroxide concentrations were determined by measuring the Malondialdehyde (MDA) content in plasma following the method described by Yoshioka *et al.* (1979). Reduced glutathione (GSH) was estimated in the whole blood following the method described by Beutler *et al.* (1963). Superoxide dismutase (SOD) level in the whole blood was estimated following the method reported by Minami and Yoshikawa (1979) and catalase (CAT) activity was determined following the method described by Johansson and Borg (1988). Glutathione peroxidase (GSH-Px) and glucose-6-phosphate dehydrogenase (G6PDH) activities were assayed following the methods described by Paglia &Valentine (1967) and Deutsch (1983). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically using the methods described by Wroblewski and La Due (1956) and Karmen (1955). Alkaline phosphatase (ALP) was determined following the method described by Bowers and Mc Comb (1966) and lactate dehydrogenase (LDH) was estimated following the method described by Gay *et al.* (1968). Creatinine concentration was determined following the method described by Lustgasten and Wenk (1972).

Statistical analysis

Student's *t*-test was used for the analysis of the biochemical parameters. The data were expressed as mean ± standard error.

RESULTS AND DISCUSSION

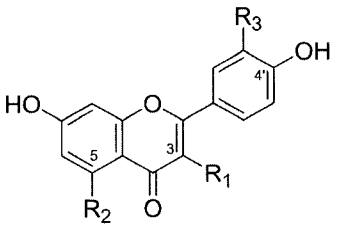
The chromatographic fractionation of 70% alcohol extract of the aerial parts of *V. calcarata* Desf. using ion exchange resin (Diaion HP-20) CC afforded 3 fractions F-1, F-2, and F-3. Fraction F-2 showed on TLC, using different stationary phases, a mixture of canary, blue and pink fluorescent

spots at 365 nm under a UV lamp giving green to greenish blue with $FeCl_3$ indicating their phenolic nature.

Column chromatography and HPLC purification of F-2 resulted in the isolation of 5 flavonol glycosides (Fig. 1). Structural identification of these compounds was carried out by acid hydrolysis, interpretation of several spectral data and by direct comparison with the data described in literature. Compounds **1~3** were identified as kaempferol-3-*O*-β-D-glucoside (Liu *et al.*, 1997), quercetin-3-*O*-β-D-glucoside (Choi *et al.*, 1998) and quercetin-3-*O*-α-L-rhamnosyl-(1→6)-β-D-glucoside (Choi *et al.*, 1998). These compounds are widespread in the plant kingdom, where as compounds **4** & **5** have rarely been seen.

On the basis of the interpretation of the spectral data of compound **4** and by comparison with values found in literature (Fuchino *et al.*, 1997), compound **4** was identified as kaempferol-3,5-*di*-*O*-β-D-diglucoside. This compound was previously found at one time in fronds of fern *Dryopteris dickinsii* (FR. Et SAV, Fuchino *et al.*, 1997). In this study, compound **4** was isolated and identified for the first time from Angiospermious plants.

The UV spectrum of compound **5** in MeOH showed absorption maxima at 265 and 350 nm. The spectral changes induced by various shift reagents suggested that compound **5** was a flavonol derivative substituted at positions 3 and 5 (Mabry *et al.*, 1970). Acid hydrolysis revealed the presence of quercetin and glucose. The HRFAB-MS (negative), high resolution FAB spectrometry, exhibited $[M-H]^+$ at m/z 625.1410 (calc.625.1405) which was in accordance with the molecular formula $C_{27}H_{30}O_{17}$, corresponding to quercetin diglucoside. Other fragments at m/z 463 and 301 suggested the presence of two glucose units. The ^{1}H-NMR signals were compatible with those of quercetin substituted by two β-glucose units with anomeric signals at δ 5.28 (1H, *d*, *J* = 7.3 Hz) and δ 4.71 (1H, *d*, *J* = 7.6 Hz). It also indicated the absence of C-5 hydroxyl group singlet of quercetin moiety. In the ^{13}C-NMR spectrum, the upfield of C-4 at δ 172.9 compared with that of compound **2**, as well as the upfield of C-3 at δ 135.3 compared with the published data of 5-*O*-glucosidated quercetin (Markham *et al.*, 1978; Tamura *et al.*, 2002) suggested the glycosylation must be at C-3 and C-5 respectively. The absence of an interglucosidic linkage between the two glucose units was clarified through the upfield of C-6" and C-6"' at δ 61.0 and δ 60.7 respectively. The 2D-NOESY experiment suggested the substitution at C-5 through the correlation between H-6 at δ 6.49 and H-1"' at δ 4.71. The HMBC experiment confirmed this finding through the correlation between the H-1"' at δ 4.71 and C-5 at δ 158.7. Therefore, from the above data, compound **5** was unequivocally determined to be quercetin-3, 5-*di*-*O*-β-D-glucoside. This compound was previously isolated at one time from a lemon peel (Chopin *et al.*, 1964). Here, we report for the first time the complete identification of this compound from a natural source.



Compound	R_1	R_2	R_3
1	*O*-Glc	OH	H
2	*O*-Glc	OH	OH
3	*O*-Glc-Rha	OH	OH
4	*O*-Glc	*O*-Glc	H
5	*O*-Glc	*O*-Glc	OH

Fig. 1. Compounds **1~5** isolated from the hepatoprotective fraction (F-2) of *Vicia calcarata* Desf. growing in Egypt

Carbon tetrachloride produces experimental liver damage that histologically resembles viral hepatitis (Laflamme, 2000). Meyer and Twedt reported that the increase in the level of serum enzymes is an indication of cellular leakage and loss of functional integrity of the cell membrane in the liver (Meyer and Twedt, 2000).

In our study, oral administration of sub-lethal doses of CCl_4 significantly ($P<0.01$) elevated the levels of the following serum enzymes; AST, ALT, ALP, and LDH in the animals of group 2 more than those of group 4 in comparison with the normal control group as shown in Table I.

The preventive action of liver damage induced by CCl_4 has widely been used as a marker of hepatoprotective activity of drugs in general (WU & Norton, 1996). Treatment of rats with F-2 during CCl_4 administration produced a

Table I. Influence of F-2 on plasma AST, ALT, ALP, and LDH activity levels in rats that received CCl_4

Groups	AST U/L	ALT U/L	ALP U/L	LDH U/L
Control	15.11±1.32 100%	5.90±1.50 100%	54.30±1.12 100%	491.41±15.5 100%
CCl_4	19.49±0.51** 129%	11.29±1.99*** 191±3.9	95.02±1.22*** 175%	1102.30±44.5*** 177%
F-2	16.27±2.15 107%	5.7.54±1.57 96.8%	61.46±2.5* 113%	523.50±21.9 105%
F-2 + CCl_4	17.67±1.34* 117%	7.39±1.11** 121%	58.10±1.11 107%	570.55±35.5* 110%

Each value is the mean of six rats.
F-2 was administrated in a dose of 25 mg/kg, body weight
Significant difference from the control group at $P < 0.05$, $P < 0.01$** & $P < 0.001$***.

significant alleviation of the hepatic injury induced by CCl_4. The hepatoprotective activity of F-2 was evaluated by its ability to lower the elevated levels of the serum enzymes resulting from CCl_4 administration. The significant reduction in enzyme levels towards respective normal values, mediated by F-2 is an indication of stabilization of the hepatocyte cell membrane as well as repairing of hepatic tissue damage caused by CCl_4. The expected mechanism of the hepatoprotective effect of F-2, against CCl_4 induced liver damage, is the ability of the flavonol glycosides content of F-2 to act as strong free radical scavengers intercepting those radicals involved in CCl_4 metabolism by microsomial enzymes. Consequently, by trapping oxygen related free radicals, the flavonol glycosides of F-2 could hinder the free radicals interaction with polyunsaturated fatty acids and would abolish the enhancement of the lipid peroxidative process leading to hepatic cell damage. The present results agree with previously reported data (Janbaz *et al.*, 2004; Hewawasam *et al.*, 2004; Wang *et al.*, 2004).

In order to verify this finding, the antioxidant activity of F-2 was determined. Lipid peroxidation content, measured as MDA, displayed a significant increase in group 2 (Table II). Also, GSH content, GSH-Px, G6PDH, SOD, and CAT activities displayed a highly significant depression in the same group (Table III). Conversely, group 4, receiving a combination of CCl_4 and F-2, displayed a significant amelioration in MDA and creatinine concentrations, as well as antioxidant parameters comparable with the values from the control group. The significant amelioration in the antioxidant parameters might result in the hepatoprotective action of flavonol glycosides against oxidative stress induced by CCl_4. Consequently, the flavonol glycosides content of F-2 is responsible for the abolishment of CCl_4-induced hepatic damage through their strong antioxidant activity. The result of the antioxidant effects of F-2 are recorded in Table III. The normal liver function parameters and the lipid peroxide content were not significantly affected in the group of animals that received F-2 alone, as shown in Tables I, II, and III. Lipid peroxidation may be prevented at the initiation stage by free radical scavengers, while the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants e.g. flavonoids (Takahama, 1983). Several studies have reported the inhibitory effects of quercetin, and other flavonoids on lipid peroxidation (Husain, *et al.*, 1987; Videla, *et al.*, 1985). Lipid peroxidation could be inhibited by flavonoids possibly by acting as strong O_2^- scavengers and $^1O_2$ quenchers. However, O_2^- itself does not appear to be capable of initiating lipid peroxidation, HO_2^- (the protonated form of O_2^-) appears to do so in isolated polyunsaturated fatty acids (Halliwell & Chirico, 1993).

Van Acker *et al.* discussed the structural aspects of the antioxidant activity of flavonoids. All in all, quercetin appears to be an extremely efficient effective radical scavenger. The strong inhibitory effect of quercetin was thought to be attributed to its additional phenolic group (3-OH). Also, kaempferol is a very good scavenger, even though it only has one hydroxyl group on the B ring (4'-OH), possibly

Table II. Influence of F-2 on plasma creatinine and malondialdehyde (MDA) contents in rats that received CCl_4

Groups	Control	CCl_4	F-2	F-2 + CCl_4
Creatinine mg/dL	1.18±0.03 100%	0.95± 0.01*** 80.3%	1.23±0.04 104%	1.32±0.03*** 111.3%
MDA (nmol/mL)	77± 0.53 100%	113±2.18*** 146.8%	80±1.44 101.9%	90± 2.38 116.9%**

Each value is the mean of six rats.
F-2 was administrated in a dose of 25 mg/kg, body weight
*Significant difference from the control group at $P < 0.01$** & $P < 0.001$***.

Table III. Influence of F-2 on blood glutathione (GSH) content and activity levels of glutathione peroxidase (GSH-Px), glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD) and Catalase (CAT) in rats that received CCl_4

Groups	GSH mg/dL	GSH-Px U/mL	G-6-PDH U/gHg	SOD U/mL	CAT μU/gHg
Control	78.47±0.60 100%	65.50±0.34 100%	3.14±0.18 100%	7.79±0.1 100%	24.57±0.91 100%
CCl_4	53.22±0.18*** 67.80%	42.54±0.9*** 61.86%	2.12±0.15*** 67.51%	5.65±0.9* 72.52	17.92±0.24** 72.93%
F-2	75.58±0.51 96.30%	64.45±0.5 98.39%	2.99±0.19 95.22%	7.71±0.4 98.97	22.91±0.4 93.24%
F2 + CCl_4	73.13±0.3*** 93.20%	60.25±0.4*** 92.75%	2.42±0.12** 87.16%	7.08±0.2 91.00	22.23±0.53 90.5%

Each value is the mean of six rats.
F-2 was administrated in a dose of 25 mg/kg body weight.
Significant difference from the control group at $P < 0.05$, $P < 0.01$ **& $P < 0.001$***.

due to the combination of other characteristics such as, a C_2-C_3 double bond, 3-OH group, and 4-keto group on ring C (Van Acker *et al.*, 1998& 1996). The mechanism of anti-radical action of quercetin and its glycoside, was evaluated by Afanas'ev *et al.* and Albano *et al.* who postulated that the inhibitory effects of both quercetin and rutin were more pronounced on CCl_4-dependent lipid peroxidation in rat liver microsomes, the activation of CCl_4 involves cytochrome P450 and does not require iron ions (Afanas'ev *et al.,* 1989; Albano *et al.*, 1982). A much stronger inhibitory effect of the flavonoids on NADPH-dependent peroxidation was ascribed to their metal-chelating properties. The flavonoids were reported to chelate iron ions and to form inert complexes unable to initiate lipid peroxidation. Yet, they retained their free radical-scavenging properties (Afanas'ev *et al.,* 1989). Van Acker *et al.* and Afanas'ev *et al.* surmised that quercetin and rutin were able to suppress free radical processes by inhibiting the formation of O_2^-, $^{\cdot}OH$, and lipid peroxyl radicals (Van Acker *et al.*, 1996&1998; Afanas'ev *et al.,* 1989).

Therefore, the data reported herein reveal a protective potential of quercetin and kaempferol glycosides, the main constituents of F-2, against the acute hepatotoxicity induced by CCl_4 in rats. Thus, this hepatoprotection could be attributed, at least in part, to the antioxidant effect of the quercetin and kaempferol glycosides, the major phytoconstituents of F-2.

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