Development and Validation of a High-Performance Liquid Chromatography Method for Standardization of the Bioactive Ethyl Acetate Fraction of *Alstonia scholaris* (Linn.) R. Br. Growing in Egypt

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Bio-guided fractionation of the ethanolic extract of the leaves of *Alstonia scholaris* (Apocynaceae) growing in Egypt was carried out to evaluate its antihyperglycemic activity in alloxan-induced diabetic rats and its hepatoprotective activity against CCl_4-induced hepatotoxicity in rats. The ethyl acetate fraction of the ethanolic extract showed the highest antihyperglycemic [(133.6 ± 4.2) mg/mL, relative to metformin with (92.3 ± 2.7) mg/mL] and hepatoprotective [(37.9 ± 1.4) U/L, relative to silymarin with (29.7 ± 0.8) U/L] activities. Four compounds were isolated from this fraction, and identified by spectroscopic techniques and by comparison with reported data: caffeic acid and isoquercitrin for the first time from this plant, in addition to quercetin 3-*O*-*β*-D-xylopyranosyl (1‴→2″)-*β*-D-galactopyranoside (major compound) and chlorogenic acid. A validated reversed phase-high-performance liquid chromatography (RP-HPLC) method was developed for the standardization of the bioactive ethyl acetate fraction. The calibration curve showed good linearity ($r^2 > 0.999$) within tested ranges. The relative standard deviation of the method was less than 3% for intra- (0.4–2.0%) and inter-day (1.9–2.8%) assays. Mean recovery of the method was within the range of 98.5–102.5%. The minimum detectable concentration of the analyte (LOD) was found to be 0.04 μg/mL. This developed HPLC method was shown to be simple, rapid, precise, reproducible, robust, specific, and accurate for quality assessment of the bioactive fraction.

Key words: Validated RP-HPLC Method, Quercetin 3-*O*-*β*-D-xylopyranosyl (1‴→2″)-*β*-D-galactopyranoside, *Alstonia scholaris*

Introduction

A remarkable percentage of the drugs commonly used today came into use through the study of indigenous remedies used throughout the world. *Alstonia scholaris* (Linn.) R. Br. (syn. *Echites scholaris* L.; *Echites pala* Ham.) is a large evergreen tree belonging to the family Apocynaceae (Kirtikar and Basu, 1996). It occurs widely in the Asia-Pacific region from India and Sri Lanka, through mainland South-East Asia to northern Australia. The bark is official in the Indian, British, and French Pharmacopoeias (Arulmozhi *et al.*, 2007). An antimalarial Ayurvedic preparation containing *A. scholaris*, Ayush-64, is marketed in India (Versha *et al.*, 2003). *A. scholaris* has been drawing the attention of researchers for its wide range of biological activities, ranging from antimalarial to anticancer. The bitter stem bark has been reputed in Hindu medicine to be useful as a digestive, laxative, anthelmintic, and stomachic (Chopra, 1958; Nadkarni, 1976; Kirtikar and Basu, 1996). The leaves are also considered important for the treatment of several ailments. The tender leaves, as a poultice, are good against ulcers, whereas a decoction of the leaves is given in cases of liver congestion (Chopra, 1958).

Applying bio-guided fractionation can result in providing a guideline for the isolation of the major compounds responsible for the biological activities of *A. scholaris* (Linn.) R. Br. In continuation of the work previously carried out in our laboratory (El-Askary *et al.*, 2012), the present study included a bio-guided study of the antihyper-

glycemic and hepatoprotective activities of leaves of *A. scholaris*, for both aqueous and ethanolic extracts, with the aim of determining active fractions and elucidating the compound(s) responsible for these activities. We also aimed to develop a validated reversed phase-high-performance liquid chromatography (RP-HPLC) method for analysis of the bioactive extract, allowing quality control and standardization of this plant's preparations.

Materials and Methods

Instrumentation

A Büchi melting point apparatus Model B-545 (Sigma-Aldrich, Munich, Germany) was used for determination of melting points, which are uncorrected. UV spectra were determined in methanol on a Shimadzu 1650 PC UV spectrophotometer (Kyoto, Japan) in the region of 200–500 nm. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian VX-300 instrument (Palo Alto, CA, USA) in $CDCl_3$ and dimethylsulfoxid-d_6 (DMSO-d_6) and chemical shifts are given in δ (ppm) relative to tetramethylsilane (TMS) as internal standard. 2D-NMR spectra were carried out in DMSO and recorded on a Bruker APX-400 NMR spectrometer (Billerica, MA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C).

High-performance liquid chromatography (HPLC) analysis was carried out on an Agilent Technologies 1100 series HPLC system (Palo Alto, CA, USA), equipped with a quaternary pump and degasser (G1322A, series 1200), a variable wavelength detector (G1314A), and a manual injector (G1328A). Agilent ChemStation software was used for data acquisition and processing. Separation was carried out on a Lichrosphere reversed phase column (RP-C18, 250 mm x 4 mm ID, 5 μm; Merck, Darmstadt, Germany), preceded by a C_{18} guard column (10 mm x 4 mm ID, 5 μm), with temperature set at 25 °C. LiChrolut RP-C18 cartridges (Merck) were used for solid phase extraction (SPE). The mobile phase was acetonitrile (Sigma-Aldrich, Steinheim, Germany) (solvent A) and 0.3% aqueous *ortho*-phosphoric acid (Sigma-Aldrich) (solvent B). Gradient elution was carried out as follows: 0–5 min, linear gradient from 15–26% A in B; 5–10 min, isocratic 26% A in B; and 10–20 min, 26–40% A in B. Flow rate was 1.0 mL/min, injection volume was 20 μL, and UV wavelength was set at 325 nm.

Chemicals

Standard phenolic acids were obtained from Merck, while standards of isoquercitrin, hyperoside, chlorogenic acid, kaempferol, and quercetin were obtained from Sigma-Aldrich. Quercetin 3-*O*-β-D-xylopyranosyl (1‴→2″)-β-D-galactopyranoside (QXG) and afzelin reference standards used for HPLC were isolated and identified in the laboratories of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt. Silymarin was obtained from Sedico Pharmaceutical (6 October City, Egypt) and carbon tetrachloride from El-Gomhoreya (Cairo, Egypt). Kits for assessment of the activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were from bioMérieux (Craponne, France).

Chromatography

Silica gel G 60 (0.063–0.200 mm mesh; Merck) was used for column chromatography (CC) and silicia gel H 60 for vacuum liquid chromatography (VLC). Silica gel 100 C18 (Fluka, Buchs, Switzerland) and Sephadex LH-20 (Pharmacia, Stockholm, Sweden) were used for CC. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 plates (Fluka) using solvent systems S_1 [EtOAc/formic acid/glacial acetic acid/H_2O (100:11:11:27, v/v/v/v)] or S_2 [$CHCl_3$/MeOH/formic acid (8:2:0.1)]. TLC plates were visualized under UV light (at 254 and 365 nm) and by spraying with 2-diphenylboric acid-2-aminethyl ester (2-diphenylboranyloxy ethanamine) natural product/polyethylene glycol (NP/PEG) spay reagent (Wagner and Bladt, 1996), and fluorescing zones then detected under UV light at 365 nm.

Plant material and extraction

Leaves of *Alstonia scholaris* (Linn.) R. Br. were collected from trees in the Zoological Garden, Giza, Egypt, in November 2007. Plant identity was kindly authenticated by Dr. Therese Labib, senior specialist of plant identification at El-Orman Botanical Garden, Cairo, Egypt. A voucher specimen (2008–002) has been deposited in the herbarium of the Faculty of Pharmacy, Cairo University, Cairo, Egypt. Methods of extraction

and fractionation of the shade-dried, powdered leaves (1.5 kg) have been reported previously (El-Askary *et al.*, 2012).

Animals

Adult male albino rats of the Sprague-Dawley strain [130–150 g body weight (BW)] were obtained from the animal house of the National Research Centre, Giza, Egypt. They were kept under the same hygienic conditions and were fed standard laboratory diet. Water was supplied *ad libitum*. All animal procedures were conducted in accordance with internationally accepted principles for laboratory animal use and care, and had been approved by the Ethics Committee of the National Research Centre (No. 9–031) in accordance with recommendations for the proper care and use of laboratory animals (NIH Publication No. 80–23; revised 1978).

Assessment of antihyperglycemic activity

Diabetes mellitus was induced according to the method described by Eliasson and Samet (1969). Ethanolic and aqueous extracts of the leaves of *A. scholaris*, as well as the fractions of the ethanolic extract of the leaves (*viz*., *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and remaining water), were tested *in vivo* for their antihyperglycemic activity against alloxan (Sigma-Aldrich)-induced hyperglycemia in adult male albino rats. The animals were injected intraperitoneally (IP) with a single dose of alloxan (150 mg/kg BW) and then divided into ten groups. The first group was kept as negative control receiving saline only; the second served as positive control (diabetic rats, received a single dose of alloxan only and thereafter left untreated), while the third to ninth groups received a daily dose of each of the seven tested samples (100 mg/kg BW of each extract). The last group was given metformin (Chemical Industries Development, Giza, Egypt) orally, in a daily dose of 150 mg/kg BW, as standard drug. At the end of each study period, blood samples were collected from the retro-orbital venous plexus through the eye canthus of anaesthetized rats after an overnight fast, and serum was isolated by centrifugation. Hyperglycemia was assessed after 72 h and after 2- and 4-week-intervals using bioMérieux kits, according to the method described by Trinder (1969).

Assessment of carbon tetrachloride-induced hepatotoxicity

Liver damage in rats was induced by IP injection of 5 mL/kg BW of 25% CCl_4 in liquid paraffin (Klassen and Plaa, 1969). Rats were randomly divided into ten groups of 10 rats each. The extracts (as above) were tested *in vivo* for their hepatoprotective activity. Group I served as control, receiving only saline, while group II served as the CCl_4-treated control group. Groups III–IX were pretreated with a daily oral dose of 100 mg/kg BW of each extract under investigation for one month. Administration of the extracts was continued after induction of liver damage by CCl_4 for another month. Group X rats were pretreated with a daily oral dose of 25 mg/kg BW of silymarin (reference drug), and administration of the drug was continued as for the extracts. Blood samples were collected as described above 30 d after CCl_4 injection. Serum ALT, AST (Thefeld *et al*., 1994), and ALP (Kind and King, 1954) were assayed according to standard methods.

Statistical analysis

Data were statistically analysed using Student's *t*-test (Snedecor and Chochran, 1971). All values are expressed as means ± SE of 10 measurements. Results with $p < 0.01$ were considered statistically significant.

Isolation of the components of the active ethyl acetate fraction

The ethyl acetate fraction (10.3 g) of the leaves was chromatographed on a VLC column (4 cm x 10 cm) filled with silica gel H 60 (100 g). Gradient elution was carried out using 100% CH_2Cl_2, then increasing the polarity up to 100% EtOAc, followed by EtOAc/MeOH up to 10% MeOH, then 100% MeOH. Fractions, each of 150 mL, were collected and monitored by TLC, and similar fractions were pooled. Fraction I (12 mg) was screened against several reference standards and was shown by co-chromatography to be caffeic acid. Fraction II (2.5 g) was further purified on a VLC column packed with RP-C18 (5 cm x 4 cm) and eluted with 15–100% aqueous MeOH. Fractions of 100 mL each were collected, affording three main fractions, subfractions II-1, II-2, and II-3. Subfraction II-1 (fraction 1, 1.5 g, eluted with 15% MeOH/H_2O) was further puri-

fied on a Sephadex LH-20 column (11 cm x 2 cm), eluted with 80% aqueous MeOH, and then re-chromatographed three times on VLC RP-C18 columns, using various gradients of MeOH/H_2O, to give chlorogenic acid (18 mg, R_f = 0.72 in S_2). Subfraction II-2 (combined fractions 3 and 4, 800 mg, eluted with 25–30% aqueous MeOH) was repeatedly purified on a silica gel column using gradient elution with EtOAc/MeOH/H_2O (95:1:2 up to 95:8:2, v/v/v) to yield the major compound (237 mg, R_f = 0.46 in S_1). Subfraction II-3 (fraction 5, 800 mg, eluted with 50% aqueous MeOH) was purified twice on a Sephadex LH-20 column using 75% aqueous MeOH to yield isoquercitrin (4 mg, R_f = 0.32 in S_1). Identification of the isolated compounds was carried out by analysis of their spectroscopic data: UV, ^{1}H NMR, and ^{13}C NMR, and the structure of the major compound was confirmed using 2D-NMR data (HMBC, HMQC, and COSY).

Caffeic acid: Greyish white powder. – R_f 0.89 (TLC, S_2). – M.p. 209–211 °C. – Colour spot in UV: blue fluorescence; NH_3/UV: green; NP-PEG: blue fluorescence under UV (365 nm); $FeCl_3$: blue. – UV (MeOH): λ_{max} = 227, 289sh, 328 nm.

Chlorogenic acid: White powder. – R_f 0.72 (TLC, S_2). – M.p. 326–329 °C. – Colour spot in UV: blue fluorescence; NH_3/UV: green; NP-PEG: blue fluorescence under UV (365 nm); $FeCl_3$: dark blue. – UV (MeOH): λ_{max} = 332, 220 nm. – ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.42 (1H, *d*, *J* = 15.9 Hz, H-7'), 7.04 (1H, *d*, *J* = 1.5 Hz, H-2'), 6.98 (1H, *br d*, *J* = 8.1, H-6'), 6.76 (1H, *d*, *J* = 8.1 Hz, H-5'), 6.16 (1H, *d*, *J* = 15.9 Hz, H-8'), 5.09 (1H, *m*, H-3), 3.92 (1H, *m*, H-5), 3.55 (1H, *dd*, *J* = 7.2, 2.4 Hz, H-4), 1.78 – 2.03 (4H, *m*, H-2 and H-6).

*Quercetin 3-O-β-*D*-xylopyranosyl (1‴→2")-β-*D*-galactopyranoside* (major compound): Yellow amorphous powder. – R_f 0.46 (TLC, S_1). – M.p. 179–181 °C. – UV (MeOH): λ_{max} = 257, 266sh, 358; (+NaOMe) 263, 378.5; (+$AlCl_3$) 274.5, 438.5; (+$AlCl_3$/HCl) 269, 300sh, 356sh, 400; (+NaOAc) 267, 409.5; (+CH_3COONa/H_3BO_3) 264, 378 nm. – ^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.69 (1H, *br s*, 5-OH), 7.77 (1H, *dd*, *J* = 8.6, 2.0 Hz, H-6'), 7.53 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.83 (1H, *d*, *J* = 8.6 Hz, H-5'), 6.39 (1H, *d*, *J* = 2.1 Hz, H-8), 6.18 (1H, *d*, *J* = 2.1 Hz, H-6), 5.69 (1H, *d*, *J* = 7.5 Hz, H-1"), 4.56 (1H, *d*, *J* = 7.0 Hz, H-1‴), 3.75 (1H, *m*, H-2"), 3.67 (1H, *m*, H-5‴), 3.65 (1H, *m*, H-3‴), 3.61 (1H, *m*, H-5"), 3.40 (2H, *m*, H-6"), 3.30 (1H, *m*, H-4"), 3.25 (1H, *m*, H-4‴), 3.10 (1H, *m*, H-2‴), 3.05 (1H, *m*, H-3"), 3.0 (1H, *m*, H-5‴). – ^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.3 (C-4), 164.2 (C-7), 161.2 (C-5), 156.1 (C-9), 155.2 (C-2), 148.5 (C-4'), 144.9 (C-3'), 133.0 (C-3), 122.2 (C-6'), 121.1 (C-1'), 115.7 (C-2'), 115.2 (C-5'), 104.6 (C-1‴), 103.7 (C-10), 98.6 (C-6), 98.3 (C-1"), 93.3 (C-8), 79.8 (C-2"), 76.1 (C-2‴), 75.8 (C-3‴), 73.9 (C-3"), 73.6 (C-5"), 69.4 (C-4‴), 67.7 (C-4"), 65.6 (C-5‴), 59.7 (C-6"). – Selected HMBC correlations for this compound are shown in Fig. 1.

Isoquercitrin: Yellow powder. – R_f 0.32 (TLC, S_1). – M.p. 230 °C. – UV (MeOH): λ_{max} = 255, 269sh, 362; (+NaOMe) 278, 327, 407; (+$AlCl_3$) 278, 303sh, 432; (+$AlCl_3$/HCl) 275, 366sh, 405; (+NaOAc) 278, 327sh, 398; (+CH_3COONa/H_3BO_3) 265, 289sh, 385 nm. – ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.71 (1H, *d*, *J* = 2.2 Hz, H-2'), 7.59 (1H, *dd*, *J* = 8.5, 2.2 Hz, H-6'), 6.87 (1H, *d*, *J* = 8.5 Hz, H-5'), 6.38 (1H, *d*, *J* = 2.1 Hz, H-8), 6.20 (1H, *d*, *J* = 2.1 Hz, H-6), 5.23 (1H, *d*, *J* = 7.50 Hz, H-1"), 3.20 – 3.73 (6H, *m*, H-2", H-3", H-4", H-5", H-6").

Sample preparation for standardization of A. scholaris extracts by HPLC

Powdered leaves (5 g) were exhaustively extracted with 3 x 20 mL MeOH by ultrasonication for 10 min each and filtered. The filtrate was evaporated to dryness under vacuum to yield a dark green residue. This residue was dissolved in 4.5 mL H_2O/0.5 mL MeOH and fractionated with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The combined ethyl acetate extract was then evaporated to dryness using a rotary evaporator. Two mg of the ethyl acetate fraction were dissolved in 5 mL of methanol in a volumetric flask and ultrasonicated. Purification was performed using solid phase extraction (SPE) cartridges (LiChrolut RP-18; Merck), which were activated and pre-conditioned using 3 x 1 mL of methanol followed by 3 x 1 mL of water. Purification was then carried out by applying the dissolved extract onto the SPE cartridge, followed by elution with H_2O/MeOH (1:4). For validation studies, a standard stock solution (0.2 mg/mL) of QXG was prepared by dissolving 5 mg of standard QXG (isolated and identified from *A. scholaris* during the course of this study) in 25 mL of methanol in a volumetric flask and ultrasonication. Methanolic solutions of the reference standards were used for spiking experiments.

Validation parameters

The method developed was validated for linearity, precision, accuracy, selectivity, limits of detection and quantification according to ICH guidelines (ICH, 2005). Linearity was determined by injecting five different concentrations of QXG standard solution (0.05 – 0.2 mg/mL), each analysed in triplicate. The calibration curve was constructed by plotting peak areas versus concentration. Data points were fitted into a line of best fit by linear regression. Accuracy was calculated as percent recovery of spiked ethyl acetate sample with QXG standard solutions at three concentrations (0.1, 0.12, and 0.2 mg/mL). Spiked samples were analysed in duplicate and accuracy expressed as mean percentage recovery at the concentrations examined. Percentage of relative standard deviation (%RSD) was calculated for each. To establish intra-day and inter-day precision of the method, repeatability (intra-day variability) was estimated by injecting two concentrations (0.12 and 0.16 mg/mL) of the standard QXG in six replicates during a single day. Intermediate precision (inter-day variability) was determined by analysing, in triplicate, the same solutions employed in the repeatability test on three consecutive days. Precision was expressed in terms of relative standard deviation (RSD). Limits of quantitation and detection (LOQ and LOD) were estimated experimentally by injecting dilutions of the standard stock solution of QXG until the signal-to-noise ratio for the standards reached 3:1 for LOD and 10:1 for LOQ. LOQ can be estimated from LOD data using the expression: LOQ = 3.3 LOD. Preliminary testing of robustness was done by making small, deliberate variations in the flow rate employed, from 1 mL/min to 0.9 and 1.1 mL/min, and %RSD was calculated (standard solution concentration was 0.16 mg/mL). Stability of the sample ethyl acetate solution was tested after 0, 6, and 36 h (kept at 4 °C before analysis).

Results and Discussion

We had previously reported the safe use of *A. scholaris*: the LD_{50} value calculated for the ethanolic extract in male albino mice, weighing 25–30 g, over 24 h was 6.8 mg/kg BW which indicates the safety of the plant (WHO, 1991; El-Askary *et al.*, 2012). The antihyperglycemic activity of a daily dose (100 mg/kg BW) of the ethanolic leaf extract after four weeks of IP administration was determined as 50.9% reduction in blood glucose level relative to the diabetic untreated animals (negative control), which was higher as compared to the aqueous extract (Table I). Consequently, the ethanolic extract was successively fractionated, and the antihyperglycemic activity in the tested fractions (daily dose of 100 mg/kg BW, treatment as above) was found to be in the following order: ethyl acetate (49.7% reduction), *n*-butanol, water, chloroform, and *n*-hexane fraction, respectively. The standard drug metformin (daily dose of 150 mg/kg BW, treat-

Table I. Antihyperglycemic activity of different extracts and fractions of the leaves of *A. scholaris* on blood glucose levels in adult male albino rats ($n = 10$).

Tested sample	Zero time	2 weeks		4 weeks	
	M ± SE [mg/mL]	M ± SE [mg/mL]	% of change	M ± SE [mg/mL]	% of change
Negative control	82.4 ± 1.6	84.1 ± 2.3	–	81.3 ± 1.9	–
Positive control	246.5 ± 9.2	256.3 ± 10.1	4.0	261.5 ± 8.9	6.1
Aqueous leaf extract	257.6 ± 11.3	193.8 ± 5.3[a]	24.8	142.4 ± 4.7[a]	44.7
Ethanolic leaf extract	261.4 ± 13.2	183.7 ± 6.1[a]	29.7	128.2 ± 4.5[a]	50.9
n-Hexane fraction	254.6 ± 8.7	231.7 ± 6.9	8.9	216.9 ± 5.8[a]	14.8
Chloroform fraction	260.3 ± 8.9	247.6 ± 7.4	4.8	221.6 ± 6.5[a]	14.9
Ethyl acetate fraction	265.8 ± 9.6	179.2 ± 5.9[a]	32.6	133.6 ± 4.2[a]	49.7
n-Butanol fraction	256.8 ± 7.9	204.2 ± 5.3	20.5	168.3 ± 3.6[a]	34.5
Water fraction	249.7 ± 8.2	193.5 ± 6.1[a]	22.5	171.2 ± 4.3[a]	31.4
Metformin	267.2 ± 9.6	139.7 ± 4.3[a]	47.7	92.3 ± 2.7[a]	65.5

M, mean of blood glucose levels. Fractions are those of the ethanolic extract. Applied doses are given in Materials and Methods.

[a] Significantly different from positive control group at $p < 0.01$.

ment as above) produced a reduction of 65.5%. All recorded values were found to be statistically significant. Results were recorded four weeks after treatment, which indicates that the herb has a moderately persistent antihyperglycemic activity on long-term continuous treatment.

All serum biochemical parameters (AST, ALT, and ALP) were significantly elevated ($p < 0.01$) in CCl_4-intoxicated rats compared with the control group, indicating acute hepatic injury. In the groups treated with the various extracts, these biochemical markers of hepatotoxicity were found to be significantly restored compared to the CCl_4-treated control group. Evidently, the ethanolic extract of the leaves of *A. scholaris* exhibited promising activity, since it caused the most significant decrease in the levels of the three enzymes. The relative potency of this total extract at 100 mg/kg BW was calculated as 95.2%, 95.8%, and 92.6% for AST, ALT, and, ALP, respectively, and therefore can be considered almost as effective as the standard silymarin at 25 mg/kg BW. Fractions of the most active extract were tested, again at 100 mg/kg BW, of which the ethyl acetate fraction exhibited the highest activity (Table II), with a relative potency of 93.5%, 90.5%, and 80.0% for AST, ALT, and ALP, respectively. These results clearly demonstrate the hepatoprotective effect of the leaves of *A. scholaris*. Moreover, the crude ethanolic extract of the leaves performed better as a hepatoprotective agent than its fractions, probably due to the synergistic effect of its constituents, or an additive effect of the different hepatoprotective constituents in the different fractions of the extract. Since the ethyl acetate fraction exhibited the best antihyperglycemic and hepatoprotective activities, further purification of the ethyl acetate fraction was deemed of interest to determine the compound(s) responsible for the observed bioactivities.

Table II. Hepatoprotective activity of different extracts and fractions of the leaves of *A. scholaris* on CCl_4-induced liver-damaged adult male albino rats.

Treatment	ALT [U/L]	AST [U/L]	ALP [KAU]
Control	31.5 ± 0.6	39.2 ± 0.8	7.2 ± 0.1
CCl_4-treated rats	181.3 ± 6.5	156.9 ± 6.8	37.1 ± 0.2
Aqueous leaf extract	34.7 ± 1.4[a]	41.2 ± 1.6[b]	10.2 ± 0.9[b]
Ethanolic leaf extract	30.6 ± 1.1[a]	35.8 ± 1.4[b]	9.3 ± 0.7[b]
n-Hexane fraction	98.3 ± 3.1[a]	95.2 ± 3.8[b]	32.8 ± 1.1[b]
Chloroform fraction	83.2 ± 3.1[a]	72.9 ± 3.6[b]	28.2 ± 0.9[b]
Ethyl acetate fraction	38.9 ± 1.1[a]	37.9 ± 1.4[b]	13.1 ± 0.4[b]
n-Butanol fraction	56.6 ± 2.1[a]	45.3 ± 1.5[b]	17.3 ± 0.6[b]
Water fraction	59.8 ± 1.3[a]	48.5 ± 1.2[b]	21.2 ± 0.7[b]
Silymarin	24.2 ± 0.5[a]	29.7 ± 0.8[b]	7.1 ± 0.2[b]

Data are expressed as means ± SE, 30 days after CCl_4 injection; $n = 10$. Applied doses are given in Materials and Methods.
[a] Statistically significant from zero time at $p < 0.01$.
[b] Statistically significant from 72 h after CCl_4 injection at $p < 0.01$.

The ethyl acetate fraction afforded four phenolic compounds. Caffeic acid and isoquercitrin have been isolated for the first time from *A. scholaris*. Chlorogenic acid has been previously reported to be present in the seeds of this plant (Baliga, 2010), but this is the first report of its isolation from the leaves. Identification of compounds was accomplished by examination of their physical and spectroscopic data (^{1}H NMR, ^{13}C NMR, 2D-NMR, and UV) and was supported by comparison with previously published data (Dürüst *et al.*, 2001; Shoeb *et al.*, 2007; Markham and Andersen, 2006; Grayer *et al.*, 2002; Larsen *et al.*, 1982; Agrawal, 1989; El-Askary, 1999; Hermann, 1978; Wagner and Blad, 1996). Identification was confirmed by co-chromatography with authentic reference standards in various solvent systems. The major compound of the ethyl acetate fraction appeared as a yellow-orange spot upon spraying with natural product reagent, indicating its flavonoidal nature. UV absorption data with different shift reagents suggested the presence of a 5,7,3',4'-tetrahydroxyflavone structure (Markham and Andersen, 2006). HMBC correlation of the terminal xylose H-1''' signal with the galactose C-2" signal, and the typical downfield shift experienced by C-2" of the primary galactose sugar unit, both confirmed 1→2 inter-glycosidic linkage. Selected HMBC correlations are shown in Fig. 1. Based on 1D- and 2D-NMR spectra, this compound was identified as quercetin 3-*O*-*β*-D-xylopyranosyl (1'''→2")-*β*-D-galactopyranoside (QXG). HMQC allowed association of most protons with the corresponding carbon signals. The 2D-NMR data are in good agreement with previously published data (Grayer *et al.*, 2002). This compound has been previously isolated from *A. scholaris* growing in Thailand (Jong-Anurakkuna *et al.*, 2007).

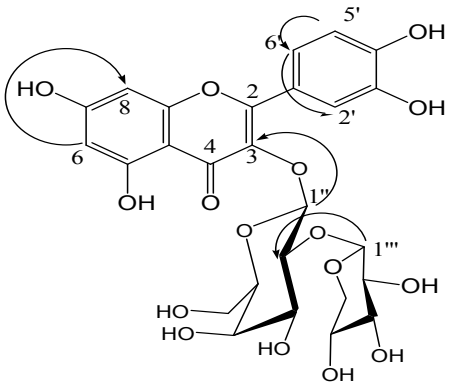

Fig. 1. Selected HMBC correlations for the major compound.

The active ethyl acetate fraction was further characterized using HPLC. Optimum chromatographic conditions, using an RP-C18 column, involved the use of acetonitrile in preference to methanol since it resulted in improved separation and an accurate HPLC fingerprint of the ethyl acetate extract. Spiking the extract with authentic standards revealed the presence of five major compounds, namely chlorogenic acid, QXG, hyperoside, isoquercitrin, and afzelin (Fig. 2). Table III lists the retention times and relative peak areas of this major constituents. The bioactivity of the ethyl acetate fraction could therefore be attributed to its high flavonoidal and phenolic acid contents. Given the predominance of QXG present in the extract (33.6% of total peak area, R_t = 6.8 min), in addition to its good resolution from other peaks, it was chosen as a marker for standardization of the extract. The calibration curve of QXG showed good linearity within the tested range, with a correlation co-efficient (r^2) of 0.999 (data not shown). Percentage recovery was within the range of 98.5–102.5%, establishing the accuracy of the method. Intra- and inter-day variation showed low relative standard deviation values within a single day (RSD 0.4–2.0%) while inter-day variation RSD values ranged between 1.9–2.8%, revealing that this method is precise. The minimum concentrations at which the analyte can be reliably detected (LOD) and quantified (LOQ) were found to be 0.04 and 0.13 μg/mL, respectively. Robustness of the proposed method was established by making small, deliberate variations in the flow rate employed, from 1 mL/min to 0.9 and 1.1 mL/min, and %RSD calculated. The chromatographic elution pattern remained unaffected and the low values of %RSD (0.747 and 0.99) established the robustness of the method. The QXG peak was well-resolved and did not interfere with any other peak in the extract samples (Fig. 2), which

Table III. Identity of the main compounds of the ethyl acetate extract under final optimized conditions.

Identified compound	R_t [min]	Peak area (%)
Chlorogenic acid	4.839	6.3
Quercetin 3-*O*-*β*-D-xylopyranosyl (1'''→2")-*β*-D-galactopyranoside (QXG)	6.802	33.6
Hyperoside	7.788	10.4
Isoquercitrin	7.946	19.9
Caffeic acid	8.742	5.34
Afzelin	11.860	15.4

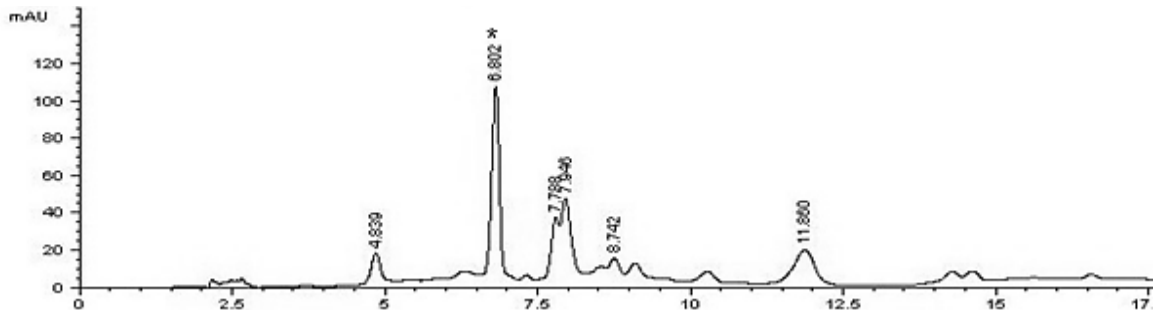

Fig. 2. HPLC chromatogram (325 nm) of the ethyl acetate fraction of the EtOH extract of leaves of *A. scholaris*. The peak marked with * represents the marker used [quercetin 3-*O*-*β*-D-xylopyranosyl (1'''→2")-*β*-D-galactopyranoside].

indicated the specificity of the proposed method. This is the first validated quantitative assay using RP-HPLC for the determination of components of the ethyl acetate fraction in *A. scholaris*. Sample stability was determined by leaving the solution at 4 °C for 36 h. The RSD values of relative peak areas found were less than 1.56%, respectively, indicating stability of the sample during this period.

Conclusion

Applying bio-guided fractionation, the compounds in the bioactive ethyl acetate fraction of *A. scholaris* (Linn.) R. Br. cultivated in Egypt were isolated. A standardized extract of the bioactive fraction was prepared using a validated HPLC method. The proposed method is linear, accurate, precise, reproducible, robust, and selective.

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