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Artigo

THE PHENOLIC COMPOSITION OF THE HEPATOPROTECTIVE AND ANTIOXIDANT FRACTIONS OF *Albizia lebbeck* L.

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An investigation of the hepatoprotective and antioxidant activities of the chloroform, ethyl acetate and *n*-butanol fractions of the leaves of *Albizia lebbeck* L. was performed. The first two fractions expressed the best results regarding the suppression of the increased levels of plasma amino-transferases and alkaline phosphatase in liver-damaged mice (after intoxication with CCl_4) compared with silymarin and the significantly increased GSH content of alloxan- induced diabetic rats compared with vitamin E (tests and reference drugs were orally administered). The bioactive fractions of *Albizia lebbeck* L. were subjected to chromatographic analysis to investigate their phenolic contents using a HPLC-PDA-ESI-MS/MS technique in the negative ion mode. The results constitute the first report of the presence of seven compounds in the genus *Albizia*, three of which were identified as 3-*O*-caffeoylquinic acid, cafeic acid, myricetin; four other flavonoids (in mg 100 g^{-1} dry powder ± SD), myricetin 3-*O*-rhamnoside (0.129 ± 0.0052), quercetin 3-*O*-dideoxypentoside (0.011 ± 0.001), kaempferol 3-*O*-glucoside (0.015 ± 0.002), quercetin 3-*O*-dihexoside (0.138 ± 0.002)*;* quercetin 3-*O*-rutinoside at a level of 0.135 ± 0.004; and the aglycones quercetin, luteolin and kaempferol. Method validation was performed, providing an analytical technique that can be used to detect trace amounts of the identified compounds in *Albizia* extracts with rapid sample preparation.

Keywords: *Albizia lebbeck*; hepatoprotection; antioxidant; phenolics; LC-PDA-ESI-MS/MS

INTRODUCTION

The *Albizia lebbeck* L. tree is native to tropical Africa, Asia, and northern Australia and is widely planted and naturalized throughout the tropics. It occurs as a popular ornamental tree in Egypt and is described as a potent herbal drug.[1] The tree is traditionally used in the treatment of ophthalmia, bronchial asthma and other allergic disorders, including chronic cough and bronchitis.[2] Biologically, *Albizia lebbeck* L. has anticonvulsant,[3,4] anthelmintic,[5,6] antioxidant,[7] antifertility,[8,9] analgesic,[10] spermicidal,[2] nootropic and anxiolytic,[11] antimicrobial[12] and *in vitro* antiprotozoal[13] effects. Previous phytochemical studies have reported the presence of flavonoids,[14-18] saponins,[19-21] β-lactam derivatives, triterpenes, sterols and hydrocarbons,[16] macrocyclic alkaloids[22,23] and tannins[24] in different tissues of the tree. The flower of *A. lebbeck* L. has been used in chronic cough, helped in removing black spots, used as an anti-allergenic, for urine retention and also as a sex tonic. Analysis of the floral odor[25-27] has revealed its chemical composition.

In continuation to the work that focused on the medicinal value of the different organs of the *A. lebbeck* L. tree cultivated in Egypt,[28] an investigation of its fractions was performed, showing suppressive effects against carbon tetrachloride (CCl_4)-induced liver injury in mice and an antioxidant effect on alloxan-induced diabetic rats. In this study, we investigated the hepatoprotection and antioxidant activity of the chloroform, ethyl acetate and *n*-butanol fractions prepared from an ethanol extract of the leaves of *A. lebbeck* L. and carried out liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis to explore their phenolic contents. The occurrence of phenolic acid derivatives, myricetin, quercetin and kaempferol glycosides other than those previously published in the bioactive fractions are reported for the first time, and no research on the hepatoprotection and antioxidant properties of active fractionsof *A. lebbeck* L. has previously been carried out.

EXPERIMENTAL

Plant material

Leaves of *A. lebbeck* L. were obtained from trees grown in the Agricultural Research Center and EL-Orman garden during 2013. The taxonomical identity was kindly verified by Dr. M. Abd El Hafez, Agricultural Research Center, Giza. A voucher specimen (A-123) has been deposited in the Herbarium, Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Standards and reagents

3-*O*-Caffeoylquinic acid, caffeic acid, quercetin, luteolin, kaempferol, myricetin, quercetin 3-*O*-rutinoside, myricetin 3-*O*-rhamnoside, and kaempferol 3-*O*-glucoside were kindly supplied by the Laboratory of Phytochemistry, Natural Products Department, NODCAR, Giza-Egypt; silymarin, from Grand Pharma Co.; vitamin E (dl-α-tocopherol acetate), from Pharco Phytopharmaceutical Co., Egypt; aloxan, from Sigma Co., USA; glutathione kit, from Wok Co., Germany; and biodiagnostic kits for estimation of serum liver enzymes (AST, ALT, and ALP), from Biomerieux, France.

Solvents

Acetonitrile, methanol and formic acid were HPLC grade

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(Merck), and deionized H_2O was treated with pure aqua RC655. The chemical reagents and solvents were all of analytical grade (BDH).

Extraction of *A. lebbeck* leaves

Air-dried and defatted (using petroleum ether) powdered leaves (1 kg) of *Albizia lebbeck* L. were exhaustively extracted with 70% ethanol. The combined extract was evaporated under reduced pressure (93 g, dry residues). The residue was suspended in water and portioned successively with chloroform, ethyl acetate and *n*-butanol saturated with water to afford 12.62 g (CFL - chloroform fraction of the leaves), 7.6 g (EAFL - ethyl acetate fraction of the leaves) and 5.5 g (BFL - *n*-butanol fraction of the leaves) dry residues, respectively.

LC-MS/MS-ESI analysis

Mass detection was performed on Thermo LCQ Advantage Max ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The analysis was carried out applying the following settings: the heated capillary and voltage were maintained at 400 °C and 4 kV, respectively; the nebulizer gas was air; the curtain gas was N_2; the collision gas was He; ionization was performed in the negative mode; and the collision energy was 35%. The full-scan mass infusion was performed using a syringe pump (Hamilton syringe, 500 µL) connected to the ESI unit at a flow rate of 10 µL mL^{-1}. The total ionmapping experiment was used as a LC-MS/MS technique, in which the production scans for each parent ion were used to determine which parent ions lost a fragment to yield a particular product ion.

The HPLC analysis was performed using a PDA detector with an Intersil ODS-2 C18 column (2.1 mm × 50 mm, particle size 3 µm, Alltech). Mobile phase: (A) 0.1% formic acid-water; and (B) acetonitrile-methanol (60:40, v/v). The gradient program was 30% B (0-2 min), 50% B (2-6 min), 70% B (6-9 min), and 70% A (9-12 min) at a flow rate of 0.2 mL min^{-1} and injection volume of 20 µL. The Xcalibur software (version 1.4) linked to the instrument was used for the calculation of the corresponding concentrations.

Sample preparation for qualitative analysis of phenolics by HPLC

CFL (250 mg) was chromatographed by vacuum liquid chromatography (VLC) using silica gel and eluted with gradients of chloroform to methanol increasing the polarity with a 5% stepwise addition of methanol to give five main fractions (200 mL each): a (40 mg), b (25 mg), c (32 mg), d (34 mg) and e (28 mg) by TLC profile, performed with cyclohexane:dichloromethane:formic acid:ethyl formate (35:30:5:30, v/v) (S1) and ethyl acetate:formic acid:acetic acid:water (100:11:11:26, v/v) (S2) as mobile phases. An aliquot of each fraction (5 mg) was separately dissolved in 5 mL of methanol and filtered through a 0.45-µm membrane filter before injection.

Sample preparation for the quantitative analysis of flavonoids by LC-ESI-MS

Air-dried powdered leaves (10 g) were defatted with petroleum ether in a Soxhlet extractor for 12 h; the plant residue was extract with 70% ethanol by sonication at room temperature (1 h). The extract was filtered, concentrated under reduced pressure, suspended in 25 mL of water and fractionated between chloroform and ethyl acetate (4 x 100 mL, each). The concentrated EAFL residue was quantitatively dissolved in 5 mL of methanol and filtered through a membrane filter (0.45 pore size) before injection.

Quantitative determination of flavonoids

Compounds used as external standards were prepared at different dilutions. Rutin and kaempferol-3-*O*-glucoside were diluted to 5 mg mL^{-1} using 1/10 dilutions each. Myricetrin was diluted to 0.087, 0.87, 4.35, 8.7, 43.5, and 87 µg each. The stock solutions were stored at -20 ºC. The standard solutions were filtered through 0.45-µm filters before injection into the HPLC and diluted as necessary with methanol. Each concentration of the standards was analyzed in triplicate. Quantification of the flavonoid glycosides was achieved by the external standard method. The analytical curves were prepared with six concentrations of each standard, and each sample was analyzed in triplicate.

Quantitative determinations of rutin, quercetin 3-*O*-dideoxypentoside and quercetin 3-*O*-dihexoside were expressed as rutin; myricetrin as myricetrin; and kaempferol 3-*O*-glucoside as kaempferol 3-*O*-glucoside. The limits of detection were calculated as the concentrations corresponding to three times the signal-noise ratio. The precision test was carried out by injecting each sample solution six times. The measurements of intra- and inter-day variability were utilized to determine the repeatability of the developed method. The intra-day variability was determined by analyzing each sample three times within the same day, and the inter-day reproducibility was performed on three different days. The RSD (relative standard deviation) was considered as the measure of precision.

Sample preparation for the hepatoprotective effect

Aliquots of the residues of CFL, EAFL, and BFL of leaves (4 g, each) of *A. lebbeck* L. were separately dissolved in distilled water (20%, w/v) containing a few drops of TWEEN® 80 for antihepatotoxicity testing.

Experimental animals

Albino mice (25-30 g) and adult male albino rats of the Sprague Dawley® Strain (130-150 g) were used. The animals were kept on a standard laboratory diet and under the same hygienic conditions. Water was supplied *ad lib*.

Toxicity

The median lethal dose (LD_{50}) values of the fractions CFL, EAFL, and BFL prepared from leaves of *A. lebbeck* L. were determined according to OECD.[29]

Hepatoprotective effect

The experimental procedures were performed according to the recommendations of the Ethical Committee of the National Research Centre and followed the guidelines of the proper care and handling of animals.

The tested fractions (CFL, EAFL, BFL) of *A. lebbeck* were evaluated and compared with a standard sample of silymarin (a powerful hepatoprotective drug that was used as a positive control),[30,31] according to a previously published method.[32] Fifty adult male albino rats were randomly divided into 5 groups (10 animals each) as follows: the first group received 1 mL of saline and was kept as the reference control group; the second group received silymarin (25 mg kg^{-1} b. wt.); and groups 3-5 received the three test solutions of *A. lebbeck* L. (100 mg kg^{-1} b. wt.), respectively. The extracts and silymarin were orally administered for two weeks, followed by induction of hepatotoxicity through an intraperitoneal injection of

Table 1. Effect of CFL, EAFL, BFL prepared from *A. lebbeck* L. and silymarin on the serum

	AST (u/L)			ALT (u/L)				ALP (KAU)			
	Zero	15wks	72h	15wks	Zero	15d	72h	15d	Zero	15d	72h	15d
Control	29.5±0.7	30.1±3.1	146.7±4.6[a]	161.3±5.2[ab]	31.4±1.2	30.8±0.7	136.9±3.6[a]	153.5±4.7[ab]	6.9±0.1	6.8±0.1	44.3±1.8[a]	62.4±2.3[ab]
CFL	29.4±0.5	29.6±0.7	87.4 ±2.6[a]	64.9±2.4[ab] (25.7%)	29.7±0.6	30.2±1.3	82.9±3.1[a]	63.7±1.2[ab] (23.2%)	6.9±0.1	7.1±0.1	31.6±1.1[a]	24.8±0.7[ba] (21.5%)
EAFL	28.7±0.6	28.2±0.4	68.9±2.1[a]	37.2±1.5[ab] (46%)	32.4±1.3	30.1±1.2	61.3±2.8[a]	38.2±0.6[ab] (37.7%)	7.1±6.1	7.3±0.2	21.4±0.9[a]	14.6±0.1[ab] (31.8%)
BFL	27.6±0.4	27.4±0.6	106.1±4.2[a]	81.5±3.1[ab] (23.2%)	28.9±0.7	29.3±0.6	91.5±3.8[a]	72.4±2.3[ab] (20.9%)	7.2±0.1	7.1±0.1	34.1±1.1[a]	28.7±0.6[ab] (15.9%)
Sily.	30.8±1.1	28.3±0.9	56.5±2.6[a]	29.3±0.8[ab] (48 %)	27.5±0.6	26.7±0.4	51.3±1.7[a]	26.1±0.3[b] (49.1%)	7.1±0.1	6.9±0.1	17.3±0.7[a]	7.3 ±0.1[b] (57.9%)

[a]Statistically significant from time zero at $p < 0.01$; [b]statistically significant from 72 h after CCl_4 at $p < 0.01$. A daily dose of 100 mg kg^{-1} b. wt. of different extracts and 50 mg kg^{-1} b. wt. of silymarin.; d, day; h, hour; Sily., silymarin as a positive control; wks, weeks.

5 mL kg^{-1} of 25% CCl_4 in liquid paraffin; then, the treatments were continued for another two weeks. After an overnight fast, blood was obtained from the rat orbital venous plexus through the eye canthus of the anaesthetized animal. The blood samples were collected at time zero and after two and four weeks. Serum was isolated by centrifugation and divided for the determination of the biochemical markers aspartate amino-transferase (AST), alanine amino-transferase (ALT) and alkaline phosphatase (ALP).

Antioxidant activity

Rats were divided into 6 groups (6 animals, each). The first group was kept as a negative control and received 1 mL of saline orally. For the other groups, *diabetes mellitus* was induced by an intraperitoneal injection of a single dose of alloxan (150 mg kg^{-1} b. wt.) in each animal followed by an overnight fast.[33] The second group of diabetic rats remained untreated, the third group received vitamin E as a reference drug (7.5 mg kg^{-1} b. wt.) and the other three groups received CFL, EAFL, and BFL (100 mg kg^{-1} b. wt., orally) of leaves of *A. lebbeck* L. Blood glutathione was determined after one week.[34]

RESULTS AND DISCUSSION

Biochemical analysis

The toxicity study revealed that oral administration of CFL, EAFL, or BFL of *A. lebbeck* in doses up to 1.3 g kg^{-1} b. wt. did not cause any signs of toxicity. Previous work has discussed the hepatoprotective activity of a 70% ethanolic extract of leaves of *A. lebbeck* in experimental liver damage induced by thioacetamide (100 mg kg^{-1}) in albino rats[35,36] and it was demonstrated that the extract had a positive effect in lowering serum enzymes. In the present study, an experiment was performed to define the bioactive fractions prepared from a 70% extract of leaves after induction of liver damage using CCl_4, a highly hepatotoxic industrial chlorinated solvent. The effects of CCl_4 intoxication (Table 1) were high recorded levels of liver enzymes, (AST, ALT and ALP) 15 days after intoxication. The hepatotoxicity of CCl_4 has been reported to be due to its biotransformation by the cytochrome P450 system (CYP2E1) in the endoplasmic reticulum of the liver to generate a highly reactive trichloromethyl ($CCl_3^{\bullet}$) radical that reacts rapidly with oxygen to form a trichloromethyl peroxy ($CCl_3O_2^{\bullet}$) radical.[37] These reactive radicals possibly attack the polyunsaturated fatty acids of the endoplasmic reticulum, thereby stimulating lipid peroxidation and disrupting Ca^{2+} homeostasis,[38,39] resulting in cellular leakage, loss of functional integrity of the cell membrane and release of the hepatic enzymes.[40] Meanwhile, the oral administration of silymarin (25 mg kg^{-1} b. wt.) decreased the level of the liver enzymes AST, ALT and ALP by 48, 49.1 and 57.9%, respectively, compared to the control group. However, simultaneous treatment of liverdamaged rats with *A. lebbeck* fractions or silymarin were significantly able to preserve biochemical changes during CCl_4 intoxication and confirmed their potential hepatoprotection activity to accelerate the regeneration of parenchymal cells, which decreased in the order of silymarin > EAFL > CFL > BFL. The reduction in the serum enzymes activities revealed the stabilization of the plasma membrane and the severity of the hepatopathy.

Table 2. Effect of CFL, EAFL, and BFL of *A. lebbeck* L. leaves on the blood glutathione level of alloxan-induced diabetic rats (n=6).

Group	Blood glutathione	
	(mg %)	% Potency
Control (1 mL saline)	36.4±1.1	_
Diabetic control	21.9±0.4[a]	39.84
Diabetic + vitamin E	35.9±1.2	1.37
Diabetic + CFL	31.2±1.2	14.28
Diabetic + EAFL	35.6±0.9	2.20
Diabetic + BFL	29.7±0.6[a]	18.41

[a] Statistically significant difference from the control at $p < 0.01$. A daily dose of 100 mg kg^{-1} b. wt. of different extracts.

The results (Table 2) demonstrated that intoxication with alloxan caused a disturbance in the antioxidant defence systems and oxidative stress, as evident from a marked decrease in the GSH content of 39.84%. In addition, the depleted level of GSH may also be due to decreased synthesis or increased utilization to counteract the excess free radicals produced.[41] In our investigation, the oral administration (100 mg kg^{-1} b. wt.) of CFL, EAFL, and BFL of the leaves of *A. lebbeck* or vitamin E significantly increased the GSH content (% change from control 14.28, 2.20, 18.41, respectively), consequently preventing oxidative stress and establishing a potential therapeutic role of fractions prepared from the leaves of *A. lebbeck* in free-radical--mediated diseases in the order of vitamin E > EAFL > CFL > BFL. The antioxidant properties of the phenolics in the EAFL and CFL are related to their redox properties and their chemical structures, which allow them to act as hydrogen donors and singlet oxygen quenchers. Some of them also display a metal chelation effect, which hinders the oxidationpromoting effect of transition metals.

For this reason, our chromatographic investigation was directed towards the highly bioactive EAFL and CFL.

Phenolics analysis

Generally, the mobile phases that were mainly used in the HPLC analysis of phenolics (flavonoids and phenolic acids) are aqueous acetonitrile, aqueous methanol, or their mixtures in combination with different concentrations of an acid as a proton source as needed for ionization, *viz.* formic acid (0.1 or 0.5%), acetic acid (0.25, 0.5, 1%), trifluoroacetic acid (0.05%), phosphoric acid (2, 0.1%), ammonium acetate (10 mmol L^{-1}) or formate (10 mmol L^{-1}). In the present study, the method development was intended to provide a reliable rapid technique for the separation of 9 flavonoids using an Intersil ODS-2 C18 column and a mobile phase consisting of mixtures of water acidulated with formic acid and acetonitrile-methanol (60:40, v/v). The analysis of the phenolics in CFL and EAFL was performed on an HPLC-PDA (Figures 1 and 2), and the peak identities were further confirmed by a LC-ESI-MS*n* (*n* = 2) system in the negative mode. This analysis was used for the separation, detection and characterization of the structure of the phenolic acids and flavonoids in the bioactive fractions in which no extensive sample preparation is required, and this analysis was shown to have a higher sensitivity for the subject compounds than MS analysis in the positive mode.

In Table 3, the MS behavior of fractions (a-e) prepared by VLC of the CFL revealed the presence of phenolic acids **1** and **2**, which were positively identified by comparison with available standards. The spectrum of compound **1** showed a deprotonated molecular ion at *m/z* 353 of a monocaffeoylquinic acid, which was first detected through a loss scan of 162 μ (a caffeic acid unit) and gave a base peak at *m/z* 191 (for quinic acid). This fragmentation is typical of 3-*O*-caffeoylquinic acid (chlorogenic acid).[42] The mass spectrum of compound **2** showed a typical loss of CO_2 for caffeic acid, giving $[M–H–44]^-$ as a characteristic ion.[43]

MS*n* fragmentation of the ion $[M–H]^-$ of the standards produced a major fragment specific for flavone and flavonols at *m/z* 151 with different intensities, originating from an RDA reaction.[44] There was a major fragment for the aglycones detected in fractions (a-e) of the CFL. Compound **3** displayed an $[M–H]^-$ ion at *m/z* 301, while compounds **4** and **5** had a deprotonated ion $[M–H]^-$ at *m/z* 285. Their fragmentation patterns match with those of standards for quercetin, luteolin and kaempferol.[44] The detection of free aglycones is commonly an indication of the presence of their glycosylated forms, but no glycosylated luteolin was detected here. Compound **6** has an $[M–H]^-$ ion at *m/z* 317, and its fragmentation behavior agreed with that of a myricitin standard.[45]

The LC-MS/MS chromatogram of the EAFL (Table 4) of *A. lebbeck* L. identified five flavonoid glycosides that are *O*-glycosylated, and their fragmentation was characterized by a cleavage of the glycosidic bonds and elimination of the sugar moieties with charge retention on the aglycone (Y_0^-). The data for the retention times (R_t), UV, deprotonated molecules $[M–H]^-$ and mass fragmentation patterns (MS*n*) of the flavonoids detected in the EAFL are listed in

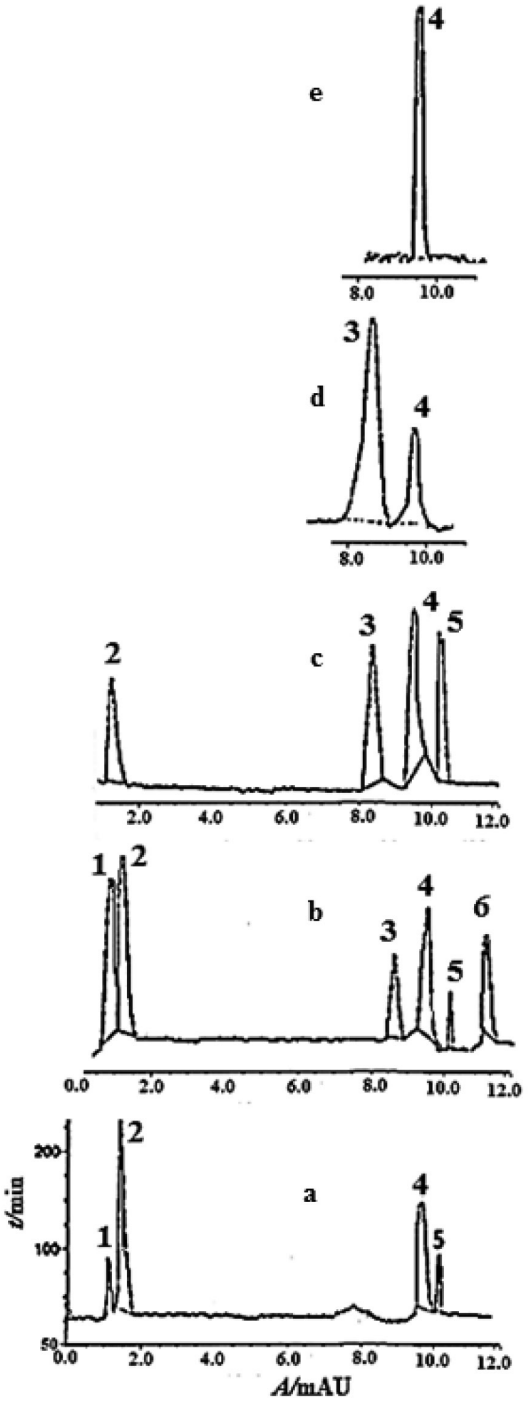

Figure 1. *HPLC/PDA chromatogram of VLC fractions (a-e) prepared from CFL of A. lebbeck*

Table 3. Retention times, UV spectra and product ions of phenolic acids and flavonoid aglycones in fractions (a-e) prepared from CFL of *A. lebbeck* L.

Peak No.	Compounds	R_t (min)	UV max (nm)	MW	MS/MS	$[M – H]^-$	Fraction				
					m/z		E	d	c	b	a
1	3-*O*-Caffeoylquinic acid	0.85	240, 298 (sh), 326	354	191, 179, 135	353	-	-	-	+	+
2	Caffeic acid	1.25	325	180	135 (100%)	179	-	-	+	+	+
3	Quercetin	8.62	256, 372	302	151 (34%)	301	-	+	+	+	-
4	Luteolin	9.64	348	286	151 (65%)	285	+	+	+	+	+
5	Kaempferol	10.45	264, 370	286	151 (55%)	285	-	-	+	+	+
6	Myricetin	11.24	254, 372	314	151 (58%)	317	-	-	-	+	-

Table 4. Retention times, UV spectra and product ions of flavonoid glycosides in EAFL of *A. lebbeck* L.

Peak No.	Compounds	R_t (min)	UV max (nm)	MW	$[M-H]^-$	LC-MS/MS *m/z*
7	Quercetin 3-*O*-rutinoside	3.88	257, 267 (sh),359	610	609	463, 447, 301([A-H]$^-$80%)
8	Myricetin 3-*O*-rhamnoside	4.65		480	479	317(60%)
9	Quercetin 3-*O*-dideoxypentoside	5.12	256, 267 (sh), 359	594	593	447(42%), 301([A-H]$^-$100%)
10	Kaempferol 3-*O*-glucoside	5.91	266, 345	448	447	285(43%)
11	Quercetin 3-*O*-dihexoside	6.42	257, 267(sh), 352	626	625	463, 301(100%)

Table 4. The ions corresponding to the deprotonated aglycone (Y_0^-) products were compared with deprotonated molecules $[M–H]^-$ of the aglycones detected in the CFL fractions; similar data are present in the literature and have been compiled for known standards that confirmed the identification of glycosides of the aglycones **3, 5, 6** as quercetin 3-*O*-rutinoside (rutin) (**7**),[16] myricetin 3-*O*-rhamnoside (**8**)[46] and kaempferol 3-*O*-β-glucoside (**10**).[47]

Because reference samples of compounds **9** and **11** (Table 4) were not available, LC- MS proved to be extremely helpful for their assignment and further characterization of individual substances with the aid of literature data. Compound **9** had a deprotonated ion $[M-H]^-$ at *m/z* 593 and (MS2, $[M-H]^-$) and other ions at *m/z* 447 (23%, $[M-146]^-$) and *m/z* 301 [100%, Y_0^-] due to the successive loss of two deoxypentoses from the aglycone quercetin (compared with compound **3**). The two sugars are attached to the same aglycone carbon. This was mainly demonstrated by comparing their peak intensities to that of rutin, indicating their link at the 3-position.[47] These ions arising from the cleavage of the glycosidic bonds are weak, and the presence of a quasi-molecular ion with low abundance (< 25) indicated the attachment of the two sugars at the same position as noticed from rutin. The flavonoid is identified as quercetin 3-*O*-dideoxypentoside. Compound **11** showed MS at *m/z* 625 $[M-H]^-$ and a peak at *m/z* 301 (100%, Y_0^-) due to the loss of two hexose moieties (324 mu) linked to the aglycone and a fragment at *m/z* 463 $[M-H-162]^-$. By comparing these fragments with those of compound **7** and other similar compounds in the literature,[46] in which the two hexoses are mostly attached to C-3, we propose the structure to be quercetin 3-*O*-dihexoside. The attachment of the sugars at this position is a recurrent characteristic of quercetin glycosides in *Albizia* species.[14,47]

Natural products derived from natural resources worldwide have a potential for protection and are successfully used to treat liver diseases.[48-50] Approximately half of the pharmaceuticals in use today are derived from natural products.[51] Their positive effects are mainly due to the presence of different phenolics *viz.*, flavonoids, coumarins, and phenolic acids.[52] The hepatoprotective effect of silymarin is mainly due to its bioactive flavonoid content.[31,53] Similarly, the promising hepatoprotective effect of *Albizia lebbeck* is mainly attributed to the flavonoids content in the fraction prepared from a 70% ethanol extract. Evidence for this reasoning is that the ethyl acetate fraction, which contains mainly flavonoid glycosides that are polyphenols with hepatoprotective and antioxidant properties, has highest activity.

Previous publications discussed the identification of quercetin and kaempferol 3-*O*-α rhamnopyranosyl (1→6)-β-glucopyranosyl (1→6)-β-galactopyranosides in leaves,[14] in addition to rutin and kaempferol-3-*O*-rutinoside in flowers[16] of *A. lebbeck*.Top of Form

To our knowledge the two phenolic acids **1** and **2**, the aglycone myricetin (**6**) and the glycosides myricetin 3-*O*-rhamnoside (**8**), quercetin 3-*O*-dideoxypentoside (**9**), kaempferol 3-*O*-glucoside (**10**), and quercetin 3-*O*-dihexoside (**11**) have not previously been reported in the genus *Albizia.* As EAFL exhibited the highest potency in the hepatotoxicity assays, it is reasonable to standardize the extracts from *Albizia* based on the contents of these major glycosides, which are probably related to the biological activity in question.

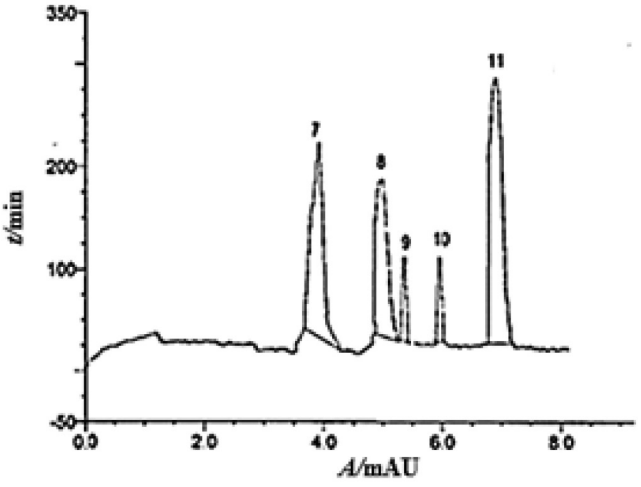

Figure 2. *HPLC/PDA chromatogram of the EAFL fraction of A. lebbeck*

Table 5. Quantitative determination of the identified flavonoids in the EAFL of *A. lebbeck*

Flavonoid glycosides	mg 100 g^{-1} dry powder ± SD[a]
Quercetin 3-*O*-rutinoside	0.135±0.004
Myricetin 3-*O*-rhamnoside	0.129±0.005
Quercetin 3-*O*-dideoxypentoside	0.011±0.001
Kampferol 3-*O*-glucoside	0.015±0.002
Quercetin 3-*O*-dihexoside	0.138±0.002
Sum of the determined flavonoid glycosides	0.428

[a] Each result is the mean of three determinations ± standard deviation.

Validation

The standard compounds quercetin 3-*O*-rutinoside (rutin), myricetin 3-*O*-rhamnoside (myricetrin) and kampferol 3-*O*-glucoside showed good linearity, with $r^2 = 0.9659, 0.9989, 0.9634$, respectively, over a relatively wide concentration range. The inter-day precision showed that the RSD (relative standard deviation) values were 2.21, 1.98, and 2.12% and the intra-day variations were 2.22, 1.688, and 1.36%, respectively. Their average regression equations were y = 415754.2 + 56122.2x (rutin); y = 314224.5 + 7112.5x (myricitrin); and y = 322235.3 + 29813.6x (kampferol 3-*O*-glucoside). The limits of detection were 2.14 (rutin), 1.22 (myricetrin), and 6.44 (kampferol 3-*O*-glycoside) ng mL^{-1}. The percentage accuracy ranged between 97.40 and 99.98%. The glycosides were quantitatively estimated (in units of mg 100 g^{-1} dry powder ± SD) as being from the EAFL

of *A. lebbeck* by LC/MS, with the results in Table 5, quercetin 3-*O*-dihexoside was present in the highest concentration (ca. 0.138 mg 100 g^{-1}) followed by quercetin 3-*O*-rutinoside (ca. 0.135 mg 100 g^{-1}).

CONCLUSION

Air-dried powdered leaves were extracted with 70% ethanol. The concentrated residue was fractionated with different organic solvents (chloroform, ethyl acetate and *n*-butanol) to yield the CFL, EAFL and BFL residual fractions, respectively. The fractions were examined for their hepatoprotection on induced liverdamaged mice and compared to silymarin as well as for their antioxidant activity on the diabetic rats compared with vitamin E. The results showed the following decreasing order of activity: silymarin > EAFL > CFL > BFL and vitamin E > EAFL > CFL > BFL, respectively. The bioactive fractions were subjected to LC-ESI-MS/MS analysis in the negative ion mode to explore their phenolics content. The results revealed the presence of 11 compounds: two phenolic acids, four flavonoid aglycones and five flavonoid glycosides. Seven of these compounds were identified here for the first time in the genus *Albizia*. Quantification of the identified flavonoid glycosides revealed that quercetin 3-*O*-rutinoside was predominant.

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