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ORIGINAL ARTICLE

Antioxidant Flavonoids from *Alhagi maurorum* with Hepatoprotective Effect

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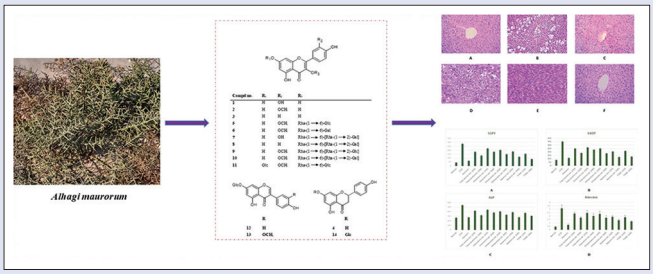
ABSTRACT

Background: *Alhagi maurorum*, commonly used in folk medicine, has been reported to have several biological activities. **Objective:** We have studied the antioxidant chemical components from *A. maurorum* to determine their *in vitro* antiproliferative and hepatoprotective activities. **Materials and Methods:** The alcoholic extract of *A. maurorum* root was subjected to a successive solvent fractionation and various chromatographic techniques guided by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay to isolate their antioxidant active compounds. The structures of the isolated compounds were identified through the extensive use of nuclear magnetic resonance and mass spectroscopy coupled with correlation to known compounds. The antioxidant and cytotoxic activities of the isolated compounds were quantified using DPPH and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays, respectively. The hepatoprotective activity of each extract and the total flavonoid fraction were assessed quantitatively on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. **Results:** Fourteen flavonoids, including four aglycones (1–4) and ten glycosides (5–14), were isolated. The flavonoid glycosides (6–14) are being reported for the first time to our knowledge. The free aglycones, those of the flavonol type, exhibited strong antioxidant and antiproliferative activities. The flavonoid glycosides exhibited weak cytotoxic activity against the hepatocellular carcinoma cell line. The total flavonoid fraction showed the strongest hepatoprotective activity against CCl_4-induced hepatotoxicity. **Conclusion:** A total of 14 flavonoids were identified from *A. maurorum*; nine of them were isolated for the first time. Flavonoids were the main chemical group identified from the *A. maurorum* root extracts, and they are responsible for the hepatoprotective activity. The findings set up a scientific explanation for the folkloric administration of *A. maurorum* in the treatment of hepatic disorders.

Key words: *Alhagi maurorum*, antioxidant, cytotoxic, flavonoids, hepatoprotective activity

SUMMARY

- A total of 14 flavonoids were identified from *Alhagi maurorum*; nine of them were isolated for the first time
- The free aglycones, those of the flavonol type, exhibited strong antioxidant activity
- The flavonoid glycosides exhibited weak cytotoxic activity against the hepatocellular carcinoma cell line
- The total flavonoid fraction showed the strongest hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity.



Abbreviations used: ^{1}H- and ^{13}C-NMR: Proton and carbon-13 nuclear magnetic resonance; CC: Column chromatography; TLC: Thin-layer chromatography; RP: Reversed phase; GAE: Gallic acid equivalent; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; QE: Quercetin equivalent; HepG2: Hepatocellular carcinoma; DMSO: Dimethyl sulphoxide; SGOT: Serum glutamate oxaloacetate transaminase; TFAM: Total flavonoids of *Alhagi maurorum*; DMEM: Dulbecco's Modified Eagle's Medium; ALP: Serum alkaline phosphatase; TB: Total bilirubin; SD: standard deviation; SGPT: Serum glutamate pyruvate transaminase; DMRT: Duncan's multiple range test; LSD: Least significance difference.

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INTRODUCTION

Alhagi maurorum Bioss. (Syn. *Alhagi camelorum*; *Alhagi pseudalhagi*) (Fabaceae) is an undershrub, glabrous, or pubescent that is 60–100 cm tall. The plant is a preferred food for camels, and thus sometimes called Shoak el-gamal, camelthorn, Agool, and Aqool.[1] It is distributed in Russia, Turkey, Iran, Syria, Afghanistan, Pakistan, and North Africa.[2] It is used in traditional medicine as an appetizer, aphrodisiac, tonic, anti-asthmatic, antirheumatic, diuretic, antipyretic, cholagogue, demulcent, and expectorant.[3] The plant has been widely used as a laxative and in treating a variety of liver disorders and various types of gastrointestinal and urinary tract disorders. Moreover, the oil from its leaves has been used in the treatment of rheumatism.[4-8] A literature survey revealed the presence of proanthocyanidins,[9] flavonoids, phenolic acids,[6,10-16] sitosterols, triterpenes,[1,10,13,17] and other chemical constituents.[18] Biological studies included antidiarrheal,[19,20] gastroprotective, anti-inflammatory, anti-ulcer, antipyretic,[6-8,21-24] antimicrobial,[25-28] antioxidant,[29] and diuretic activities.[30-32]

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In our continued search for lead compounds derived from natural sources, we worked extensively on the dried underground parts of *A. maurorum* to characterize their phenolic compounds and potential biological activities. Although various pharmacological activities have been declared for *A. maurorum* root extract, few deep phytochemical studies have been carried out to explore its main active metabolites and the corresponding biological activities. The purpose of the current study is to assess the antioxidant, cytotoxic, and hepatoprotective activities of the different extracts of *A. maurorum* and its phenolic metabolites.

MATERIALS AND METHODS

General

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR experiments were measured in dimethyl sulphoxide (DMSO)-d_6 on a Bruker Avance DRX 600 spectrometer (Bruker GmbH, Rheinstetten, Germany). Column chromatography (CC) was carried out on Sephadex LH-20 (25–100 mm mesh size, Sigma-Aldrich, Munich, Germany), Diaion HP-20 (Mitsubishi Chemical Co., Ltd., Tokyo, Japan), RP-18 (Merck, Darmstadt, Germany), and Kieselgel 60 (60–230 mesh, Merck). Purification was carried out using flash medium-pressure liquid chromatography (22 mm, i.d. × 30 cm, Kusano Scientific Co., Tokyo, Japan) and an Agilent 1100 Series separation module high-performance liquid chromatography (HPLC) equipped with Agilent 1200 Series diode array detector and an Agilent Chromatorex Zorbax SB semi-preparative C_{18} column (5 µm, 9.4 × 250 mm). Precoated silica gel RP-C_{18} F_{254S} and G60 F_{254} (E-Merck) plates were used for thin-layer chromatography (TLC), and 10% H_2SO_4 was used as a spraying reagent followed by heating for 3 min.

Plant material

The soil-free roots of *A. maurorum* Bioss were collected in 2015 from the field of Assiut governorate during summer. Prof. Dr. G. El-Naggar, Professor of Taxonomy, Botany Department, Faculty of Sciences, Assiut University, identified the plant. A voucher specimen (No. 2015-ALM) was kept in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Assiut University, as reference material.

Extraction and isolation

800 g of the powdered plant material was extracted with 70% aqueous ethanol by maceration and percolation (3 L × 3) until exhaustion. The ethanol extract was concentrated under reduced pressure to give 150 g (18.8% w/w) of brown viscous extract. Thereafter, the ethanolic extract (90 g) was blended with 400 mL of distilled water. The mixture was then taken for consecutive solvent fractionation with *n*-hexane, dichloromethane, and ethyl acetate to yield 9.2 g, 24.3 g, and 27.1 g, respectively. The dichloromethane fraction was subjected to successive normal-phase CC using a gradient $CHCl_3$–MeOH mixture. Four compounds were isolated from the CH_2Cl_2 fraction; compounds 4 (19.3 mg), 3 (24.5 mg), 2 (18.6 mg), and 1 (16.11 mg).

The ethyl acetate fraction was applied to normal-phase CC using $CHCl_3$–MeOH mixture via a gradient elution to afford four fractions (F1 to F4). Fraction F2 was subjected to medium pressure liquid chromatography (MPLC) using an RP-18 column and a CH_3CN–H_2O gradient followed by RP-HPLC using a gradient of CH_3CN–H_2O (0%–100%) over 30 min to afford compounds 12 (12.1 mg), 13 (13.6 mg), and 14 (6.8 mg).

The remaining aqueous fraction (28.1 g) was concentrated under reduced pressure and subjected to a column of Diaion HP-20 and eluted with water; 25%, 50%, and 75% methanol in water; and then 100% methanol, followed by washing with acetone to give 2.5 g (25% MeOH), 6.9 g (50% MeOH), 3.6 g (75% MeOH), and 2.9 g (100% methanol). The 50% MeOH and 75% MeOH fractions were combined (because of the similarities between them visualized on TLC) and were subjected to normal-phase CC using a $CHCl_3$–MeOH gradient affording three fractions (F1 to F3). Fractions F2 and F3 were subjected to RP-HPLC using CH_3CN–H_2O gradient elution (0%–100%) over 45 min to give compounds 11 (5.1 mg) and 9 (6.5 mg) from F2 and compounds 7 (4.7 mg), 8 (7.8 mg), and 10 (11.4 mg) from F3. The 100% methanol fraction was subjected on a Sephadex LH-20 column, followed by RP-HPLC using CH_3CN–H_2O gradient elution (0%–100%) over 20 min to give compounds 5 (22 mg) and 6 (9.7 mg).

Compound 5

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.83 (3H, *s*, OMe), 6.21 (1H, *d*, *J* = 1.5 Hz, H-6), 6.43 (1H, *d*, *J* = 1.5 Hz, H-8), 7.86 (1H, *d*, *J* = 1.4 Hz, H-2`), 6.91 (1H, *d*, *J* = 7.0 Hz, H-5`), 7.51 (1H, *dd*, *J* = 7.0, 1.4 Hz, H-6`), and 12.57 (1H, *s*, 5-OH); sugar moiety: δ_H 0.91 (3H, *d*, *J* = 6.2 Hz, H-6``` rham), 3.05–3.71 (10H, *m*, other sugar protons), 4.41 (1H, *br. s*, H-1``` rham), and 5.43 (1H, *d*, *J* = 7.5 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 6

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.85 (3H, *s*, OMe), 6.12 (1H, *br. s*, H-6), 6.34 (1H, *br. s*, H-8), 7.99 (1H, *br. s*, H-2`), 6.89 (1H, *d*, *J* = 7.0 Hz, H-5`), 7.49 (1H, *br. d*, *J* = 7.0 Hz, H-6`), and 12.54 (1H, *s*, 5-OH); sugar moiety: δ_H 1.05 (3H, *d*, *J* = 6.1 Hz, H-6``` rham), 3.07–3.71 (10H, *m*, other sugar protons), 4.42 (1H, *br. s*, H-1``` rham), and 5.42 (1H, *d*, *J* = 6.8 Hz, H-1`` gal). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 7

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 6.17 (1H, *br. s*, H-6), 6.37 (1H, *br. s*, H-8), 7.48 (1H, *d*, *J* = 1.7 Hz, H-2`), 6.80 (1H, *d*, *J* = 7.1 Hz, H-5`), 7.67 (1H, *dd*, *J* = 7.1, 1.7 Hz, H-6`), and 12.70 (1H, *s*, 5-OH); sugar moiety: δ_H 0.79, (3H, *d*, *J* = 6.1 Hz, H-6```` rham), 1.05 (3H, *d*, *J* = 6.2 Hz, H-6``` rham), 3.03–3.82 (13H, *m*, other sugar protons), 3.80 (1H, *m*, H-2 gal), 4.38 (1H, *br. s*, H-1````` rham), 5.05 (1H, *br. s*, H-1``` rham), and 5.57 (1H, *d*, *J* = 6.9 Hz, H-1`` gal). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 8

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 6.15 (1H, *br. s*, H-6), 6.38 (1H, *br. s*, H-8), 8.03 (2H, *d*, *J* = 7.4 Hz, H-2`, 6`), 6.85 (2H, *d*, *J* = 7.4 Hz, H-3`, 5`), and 12.67 (1H, *s*, 5-OH); sugar moiety: δ_H 0.77 (3H, *d*, *J* = 6.0 Hz, H-6```` rham), 1.04 (3H, *d*, *J* = 6.1 Hz, H-6``` rham), 3.08–3.73 (13H, *m*, other sugar protons), 3.77 (1H, m, H-2 gal), 4.35 (1H, *br. s*, H-1```` rham), 5.04 (1H, *br. s*, H-1``` rham), and 5.54 (1H, *d*, *J* = 6.9 Hz, H-1`` gal). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 9

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.84 (3H, *s*, OMe), 6.17 (1H, *br. s*, H-6), 6.39 (1H, *br. s*, H-8), 7.84 (1H, *d*, *J* = 1.5 Hz, H-2`), 6.89 (1H, *d*, *J* = 7.0 Hz, H-5`), and 7.48 (1H, *dd*, *J* = 7.0, 1.5 Hz, H-6`); sugar moiety: δ_H 0.70 (3H, *d*, *J* = 6.0 Hz, H-6```` rham), 0.95 (3H, *d*, *J* = 5.9 Hz, H-6``` rham), 3.03–3.72 (13H, *m*, other sugar protons), 3.45 (1H, *m*, H-2 glu), 4.37 (1H, *br. s*, H-1````` rham), 5.02 (1H, *br. s*, H-1``` rham), and 5.61 (1H, *d*, *J* = 7.4 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 10

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.87 (3H, *s*, OMe), 6.17 (1H, *d*, *J* = 1.2 Hz, H-6), 6.41 (1H, *d*, *J* = 1.2 Hz, H-8), 8.00 (1H, *d*, *J* = 1.5 Hz, H-2`), 6.89 (1H, *d*, *J* = 7.1 Hz, H-5`), 7.49 (1H, *dd*, *J* = 7.1, 1.5 Hz, H-6`), and 12.65 (1H, *s*, 5-OH); sugar moiety: δ_H 0.70 (3H, *d*, *J* = 6.0 Hz, H-6```` rham), 1.04 (3H, *d*, *J* = 6.1 Hz, H-6``` rham),

Table 1: Carbon-13 nuclear magnetic resonance data of compounds (5-14), 150 MHz in dimethyl sulphoxide-d_6

Carbon number	δ_C (multiple)									
	5	6	7	8	9	10	11	12	13	14
2	156.7 s	155.9 s	156.8 s	156.2 s	156.5 s	156.1 s	156.1 s	154.6 d	154.8 d	78.7 d
3	133.1 s	133.0 s	133.4 s	132.4 s	132.5 s	132.6 s	133.3 s	122.6 s	122.8 s	42.4 t
4	177.4 s	177.2 s	177.8 s	177.2 s	177.2 s	177.2 s	177.6 s	180.5 s	181.1 s	197.3 s
5	161.0 s	161.1 s	161.8 s	161.2 s	161.2 s	161.2 s	160.9 s	161.6 s	161.6 s	162.9 s
6	98.9 d	99.1 d	99.3 d	98.9 d	98.7 d	98.9 d	99.4 d	99.6 d	99.7 d	96.5 d
7	164.3 s	164.0 s	164.2 s	164.5 s	164.5 s	164.6 s	162.9 s	163.0 s	163.0 s	165.2 s
8	94.1 d	94.0 d	94.2 d	93.8 d	93.9 d	93.8 d	94.8 d	94.5 d	94.6 d	95.4 d
9	156.6 s	156.6 s	156.9 s	156.4 s	156.5 s	156.4 s	157.0 s	157.2 s	157.3 s	162.8 s
10	104.1 s	103.8 s	104.4 s	103.7 s	104.0 s	103.9 s	105.7 s	106.1 s	106.1 s	103.2 s
1`	121.2 s	121.0 s	121.7 s	121.0 s	121.1 s	121.1 s	120.9 s	121.0 s	121.4 s	128.6 s
2`	115.3 d	115.1 d	115.7 d	130.8 d	115.2 d	115.2 d	115.3 d	130.2 d	115.3 d	128.4 d
3`	149.5 s	149.5 s	145.5 s	115.1 d	149.4 s	149.4 s	149.7 s	115.1 d	147.4 s	115.2 d
4`	147.0 s	147.0 s	149.5 s	159.2 s	146.8 s	147.1 s	146.9 s	157.5 s	146.9 s	157.8 s
5`	113.4 d	113.4 d	116.3 d	115.1 d	113.3 d	113.4 d	113.3 d	115.1 d	113.3 d	115.2 d
6`	122.4 d	121.9 d	122.6 d	130.8 d	122.1 d	121.8 d	122.5 d	130.2 d	121.8 d	128.4 d
Sugar I	**Glc**	**Gal**	**Gal**	**Gal**	**Glc**	**Gal**	**Glc**	**Glc**	**Glc**	**Glc**
1``	101.3 d	101.9 d	99.6 d	99.4 d	98.9 d	99.0 d	101.1 d	99.8 d	100.2 d	99.5 d
2``	74.4 d	71.1 d	75.0 d	74.9 d	77.6 d	75.2 d	74.3 d	73.1 d	73.7 d	73.0 d
3``	76.5 d	73.0 d	74.0 d	73.8 d	77.0 d	73.4 d	76.4 d	77.2 d	77.2 d	77.0 d
4``	70.2 d	68.3 d	68.3 d	68.2 d	70.6 d	68.2 d	70.1 d	69.6 d	70.2 d	69.5 d
5``	76.0 d	73.5 d	73.5 d	73.3 d	75.8 d	73.7 d	76.0 d	76.4 d	76.3 d	76.4 d
6``	67.0 t	65.1 t	65.1 t	65.2 t	66.6 t	65.1 t	66.9 t	60.6 t	60.7 t	60.5 d
Sugar II	**Rha**	**Rha**	**Rha**	**Rha**	**Rha**	**Rha**	**Rha**			
1```	101.0 d	100.0 d	100.6 d	100.6 d	100.9 d	100.9 d	100.9 d			
2```	70.7 d	70.6 d	70.8 d	70.7 d	70.6 d	70.7 d	70.6 d			
3```	70.4 d	70.4 d	70.7 d	70.6 d	70.5 d	70.5 d	70.3 d			
4```	71.9 d	71.9 d	72.0 d	71.9 d	71.8 d	71.9 d	71.8 d			
5```	68.4 d	67.9 d	68.5 d	68.3 d	68.3 d	68.3 d	68.3 d			
6```	17.8 q	17.9 q	17.5 q	17.3 q	17.1 q	17.1 q	17.8 q			
Sugar III			**Rha**	**Rha**	**Rha**	**Rha**	**Glc**			
1````			99.9 d	100.1 d	100.8 d	100.1 d	99.9 d			
2````			70.7 d	70.6 d	70.6 d	70.7 d	73.2 d			
3````			70.6 d	70.4 d	70.4 d	70.6 d	77.2 d			
4````			72.0 d	71.9 d	71.8 d	71.8 d	69.6 d			
5````			68.7 d	68.6 d	68.3 d	68.6 d	76.5 d			
6````			18.0 q	17.9 q	17.7 q	17.9 q	60.7 t			
OMe	55.8 q	55.9 q	-	-	55.7 q	55.9 q	55.7 q	-	55.2 q	-

Glc: Glucose; Rha: Rhamnose; Gal: Galactose

3.06–3.76 (13H, *m*, other sugar protons), 3.80 (1H, m, H-2 gal), 4.39 (1H, *br. s*, H-1```` rham), 4.98 (1H, *br. s*, H-1``` rham), and 5.66 (1H, *d*, *J* = 6.9 Hz, H-1`` gal). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 11

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.84 (3H, *s*, OMe), 6.45 (1H, *d*, *J* = 1.4 Hz, H-6), 6.78 (1H, *d*, *J* = 1.4 Hz, H-8), 7.87 (1H, *d*, *J* = 1.6 Hz, H-2`), 6.91 (1H, *d*, *J* = 7.0 Hz, H-5`), and 7.55 (1H, *dd*, *J* = 7.0, 1.6 Hz, H-6`); sugar moiety: δ 0.97 (3H, *d*, *J* = 5.9 Hz, H-6``` rham), 3.04–3.71 (16H, *m*, other sugar protons), 4.41 (1H, *br. s*, H-1``` rham), 5.06 (1H, *d*, *J* = 7.2 Hz, H-1```` glc), and 5.46 (1H, *d*, *J* = 7.4 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 12

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 8.43 (1H, *s*, H-2), 6.46 (1H, *d*, *J* = 2.0 Hz, H-6), 6.71 (1H, *d*, *J* = 2.0 Hz, H-8), 7.40 (2H, *d*, *J* = 7.0 Hz, H-2`, 6`), 6.82 (2H, *d*, *J* = 7.0 Hz, H-3`, 5`), 9.63 (1H, *s*, 4`-OH), and 12.93 (1H, *s*, 5-OH); sugar moiety: δ_H 3.16–3.71 (6H, *m*, sugar protons) and 5.05 (1H, *d*, *J* = 7.3 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 13

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 3.78 (3H, *s*, OMe), 8.44 (1H, *s*, H-2), 6.46 (1H, *d*, *J* = 1.5 Hz, H-6), 6.71 (1H, *d*, *J* = 1.5 Hz, H-8), 7.15 (1H, *d*, *J* = 1.0 Hz, H-2`), 6.81 (1H, *d*, *J* = 6.5 Hz, H-5`), 6.99 (1H, *dd*, *J* = 6.5, 1.0 Hz, H-6`), 9.18 (1H, *s*, 4`-OH), and 12.90 (1H, *s*, 5-OH); sugar moiety: δ_H 3.14–3.70 (6H, *m*, sugar protons) and 5.05 (1H, *d*, *J* = 7.4 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Compound 14

^{1}H NMR data (600 MHz, DMSO-d_6): aglycone: δ_H 5.48 (1H, *dd*, *J* = 13.5, 2.8 Hz, H-2), 2.72 (1H, *dd*, *J* = 16.9, 2.8 Hz, H-3 eq), 3.32 (1H, *m*, H-3 ax), 6.11 (1H, *d*, *J* = 1.9 Hz, H-6), 6.13 (1H, *d*, *J* = 1.9 Hz, H-8), 7.31 (2H, *d*, *J* = 7 Hz, H-2`, 6`), 6.78 (2H, *d*, *J* = 7 Hz, H-3`, 5`), 9.61 (1H, *br. s*, 4`-OH), and 11.99 (1H, *br. s*, 5-OH); sugar moiety: δ_H 3.12–3.67 (6H, *m*, sugar protons) and 4.96 (1H, *d*, *J* = 7.5 Hz, H-1`` glc). ^{13}C NMR data (150 MHz, DMSO-d_6) [Table 1].

Acid hydrolysis of isolated flavonoid glycosides

3 mg of the isolated flavonoid glycosides was dissolved in 5 mL of HCl (2 M) and refluxed for 2 h. The reaction mixture was left to cool and then shaken with 2 mL of ethyl acetate thrice to extract the aglycone part,

leaving the sugar parts in the aqueous layer. The sugars were identified by comparison with authentic samples, adopting the procedure of Abdel-Mageed *et al.*, 2014.[33]

Determination of total phenolic content

Folin–Ciocalteu reagent method was used to ascertain the amount of total phenolics in the different extracts of *A. maurorum* leaves. In a test tube, a mixture of 0.5 mL of various *A. maurorum* extracts (1 mg/mL) was mixed with 2.0 mL of sodium carbonate (2%, w/v) and 2.5 mL of Folin–Ciocalteu reagent 10% (V/V) and then incubated with intermittent shaking at 45°C for 15 min. The absorbance was noted at 765 nm, and the calibration curve was prepared utilizing gallic acid as a standard. The results were expressed as gallic acid equivalents in milligrams per gram of dried extract.[34]

Quantitative estimation of total flavonoids

The content of total flavonoid in the different extracts of *A. maurorum* was determined based on the formation of a flavonoid–aluminum complex, adopting the procedure described by Sen *et al.*, 2013, where 0.1 mL of 1 mol/L potassium acetate, 0.1 mL of 10% aluminum nitrate, and 4.3 mL of 80% ethanol were mixed with 0.5 mL of various solvent extracts (1 mg/mL) and incubated for 40 min at 23°C until a yellow color developed indicating the presence of a flavonoid. The absorbance of each extract was measured at 415 nm, and quercetin was used as a standard material. Different concentrations of quercetin solutions were prepared to create the standard calibration curve, and the results for each extract were expressed as quercetin equivalents in milligram per gram of dried extract.[35,36]

Preparation of the total flavonoids of *Alhagi maurorum*

The alcoholic extract (20 g) was dissolved in hot water and then alkalinized with 5% sodium carbonate to pH 9–10. The process was followed by removal of lipophilic constituents by consecutive extractions with chloroform. Thereafter, hydrochloric acid (1 N) was used to acidify the remaining aqueous extract to pH 4. The total flavonoids of *A. maurorum* (TFAM) preparation was carried out adopting the procedure described by Wu *et al.*, 2006.[37] A dark brown powder (4.5 g) was obtained which was considered as the TFAM.

1,1-dipheny l-2-picrylhydrazyl radical-scavenging assay

The radical-scavenging activities of the isolated compounds were qualitatively and quantitatively assessed against 0.2% 1,1-diphenyl-2-picrylhydrazyl (DPPH)* in MeOH according to previously described methods[34,35] using ascorbic acid as a positive control.

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide cytotoxic assay

The hepatocellular carcinoma (HepG2) cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were sustained and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, and antibiotics (0.10 g/L streptomycin and 0.06 g/L penicillin – Sigma-Aldrich, Munich, Germany) at 95% humidity, 5% CO_2, and 37°C. Solutions of the isolated flavonoids were prepared at different concentrations in 0.1% DMSO solution. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assays were used to assess the *in vitro* antiproliferative action of the isolated flavonoids against HepG2, adopting the procedure described by Ge *et al.*, 2018. Doxorubicin was chosen as a positive control to evaluate cytotoxicity.[38]

Animals

Male albino rats (78 in total) of Wistar albino strain of similar age (8–10 weeks) and weight (150–200 g) were obtained from the Experimental Animal Center, College of Pharmacy, King Saud University. The conditions at which the animals were housed were kept at constant levels: humidity (55%), temperature (23°C ± 2°C), and (12/12 h) light/dark conditions. They had free access to drinking water *ad libitum* and were supplied with Purina chow. The institutional animal ethics committee approved the animal experimental protocol for this study.

Hepatoprotective activity

Thirteen groups of male Wistar rats containing six animals each were designed. Normal saline was provided to Group I and was marked as the negative control group. The other groups (II–XIII) received carbon tetrachloride (CCl_4) (0.1 mL) in liquid paraffin (1:1) intraperitoneally per 100 g body weight. Group II was administered only the CCl_4 treatment. Group III was given silymarin (Sigma, St. Louis, MO, USA) through oral administration (p.o.) at a dose of 20 mg/kg. Groups (IV-XI) were treated with 250 and 500 mg/kg of the different *A. maurorum* extracts, whereas Groups XII and XIII were treated with 150 and 300 mg/kg of the TFAM fraction, respectively. Drugs were administered 5 days prior to the CCl_4 application and were continued until completion of the experiment. Ether anesthesia was used to sacrifice the animals after 24 h of $CC1_4$ administration. Blood samples were collected and centrifuged at 3000 rpm for 5 min and then stored at −20°C for evaluation of the biochemical parameters. For the purpose of histopathological investigation, the livers were instantly removed and fixed in 10% formalin.[39]

Biochemical estimations

As per the standard protocols of Edwards and Bouchier, 1991,[40] the serum enzymatic parameters, such as alkaline phosphatase (ALP), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), as well as the nonenzymatic parameters, such as total bilirubin (TB), were assayed. Diagnostic strips (Reflotron, Roche Diagnostics, Mannheim, Germany) were used to measure the enzymatic activity reading on a Reflotron® Plus instrument (ROCHE).

Statistical analysis

A one-way analysis of variance technique was used to analyze the data statistically. The values were depicted as means ± standard deviations. Duncan's multiple range test and the least significance difference test facilitated comparison of the significance levels of the means. The level of significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.[41,42]

Histopathology

For histopathological assessments, the livers were removed instantly just after the sacrifice of the treated animals, and a small piece of each liver was fixed in 10% buffered neutral formalin for 12 h for use. All specimens were immersed in different concentrations of ethanol, which ranged from 70% to 100%, followed by xylene (3 times, 1 h and each) and finally paraffin wax (4 times, 1 h and each) for dehydration, clearing, and infiltration. Sections of thickness 3–4 μm were cut by a Leitz 1512 rotary microtome (Ernst Leitz Wetlzar, Rockleigh, NJ, USA) and were stained with Mayer's hematoxylin solution for 15 min. Thereafter, the slides were washed with distilled water and then by 80% ethyl alcohol for 1–2 min.

Finally, staining was performed with eosin-phloxine solution for 2 min. Light microscopy revealed the histopathological changes.[39]

RESULTS

The phytochemical investigation of the root extract of *A. maurorum*, guided by the findings of antioxidant radical scavenging assay, led to the isolation and identification of 14 flavonoids (1-14), including four free aglycones: quercetin (1),[43] isorhamnetin (2),[43] kaempferol (3),[44] naringenin (4)[45] and ten glycosides: isorhamnetin-3-*O*-rutinoside (5),[46] isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (isorhamnetin 3-robinobioside) (6),[47] quercetin-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl-β-D-galactopyranoside) (alcesefoliside) (7),[48] kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (mauritianin) (8),[49] isorhamnetin 3-*O*-(2,6-di-*O*-α-rhamnosyl)-β-D-glucopyranoside (typhaneoside) (9),[50] isorhamnetin 3-*O*-(2,6-di-*O*-α-rhamnosyl)-β-D-galactopyranoside (10),[51] isorhamnetin-3-*O*-rutinoside-7-*O*-glucopyranoside (11),[52] genistin (12),[53] 3'-*O*-methylorobol 7-*O*-β-D-glucoside (13),[54] and naringenin-7-glucopyranoside (14)[55] [Figure 1]. All structures were identified using extensive NMR analyses [Table 1], accurate mass measurements, and comparisons to published data.

DPPH radical scavenging assay was used to quantify the antioxidant activity of the isolated flavonoids. The findings [Table 2] reveal that all the isolated flavonoids demonstrate significant free radical scavenging activity of variable potency. The free flavonol aglycones (1–3) have the strongest antioxidant activity. Quercetin (1) was identified as the most potent flavonoid with an IC_{50} value of 10.1 μM.

For the *in vitro* antihepatoma activity, the MTT colorimetric assay was used to assess the isolated compounds against human HepG2 cells in the presence of doxorubicin as a positive control [Table 2]. Compounds **1** and **3** (quercetin and kaempferol) exhibited the strongest antihepatoma activity with IC_{50s} values of 18.9 and 35.7 μM, respectively [Table 2].

The hepatoprotective activities of different *A. maurorum* extracts and TFAM were assessed against the chemical injury in the liver cells (hepatocytes) induced by the classical hepatotoxic agent CCl_4. Hepatic toxicity induced by the intraperitoneal injection of a single dose of CCl_4 is reflected by a significant elevation of biochemical parameter such as SGPT, ALP, SGOT, and TB accompanied by severe damage of hepatic cells, biliary obstruction, and transport inability, in comparison with the control group.

Pretreatment of rats with silymarin significantly reduced the observed elevation in SGPT, SGOT, ALP, and bilirubin induced by CCl_4 (76.9, 68.0, 49.3 and 77.1%, respectively, $P < 0.001$) [Table 3], indicating a good recovery from the hepatotoxic effect of CCl_4. Pretreatment with *A. maurorum* total extract and its subfractions (chloroform, ethyl acetate, and aqueous) showed a significant reduction of the biochemical parameters in a dose-dependent manner. The maximum hepatoprotective effect was observed with the aqueous extract at a dose of 500 mg/kg with 58.8, 64.3, 48.9, and 55.6% reduction in the biochemical parameters SGPT, ALP, SGOT, and bilirubin levels, respectively ($P < 0.01$). Moreover, the oral pretreatment of rats with the TFAM significantly ameliorated the increases observed in SGPT, SGOT, ALP, and TB (69.1, 62.4, 43.6, and 60.7%, respectively) at a dose of 300 mg/kg ($P < 0.01$) [Table 3].

Based on the results observed in the biochemical parameters presented above, the livers of rats pretreated with TFAM were subjected to histological study. The histological appearance of the hepatic cells [Figure 2] showed that exposure to the hepatotoxic agent CCl_4 changes the normal histological appearance [Figure 2a], with observations of severe hepatic lesions marked by extensive fatty changes and hepatocyte necrosis [Figure 2b]. The pretreatment of rats with silymarin [Figure 2c] and TFAM [Figure 2f] showed a good recovery in the hepatocytes accompanied by the disappearance of necrosis and fatty depositions compared with ethyl acetate [Figure 2d] and aqueous extract [Figure 2e]. The results of the *in vivo* histopathological examinations proved the significant corrective effects of TFAM on the hepatic lesions induced by CCl_4, which is in agreement with the results of the biochemical tests.

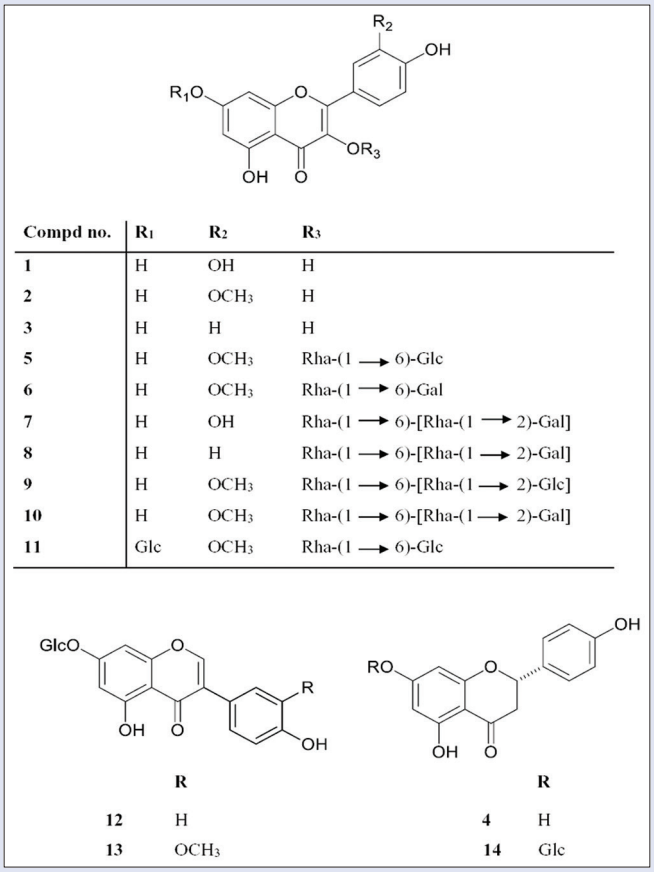

Figure 1: Chemical structures of the isolated compounds

Table 2: Antioxidant and cytotoxic activities of the isolated compounds (1-14)

Compound	IC_{50} (μM)	
	DPPH	HepG2
1	10.1	18.9
2	18.5	42.4
3	16.3	35.7
4	>100	>100
5	31.7	76.5
6	32.2	88.4
7	26.1	57.1
8	>100	>100
9	40.3	>100
10	55.8	>100
11	47.4	>100
12	>100	74.3
13	>100	52.5
14	>100	>100
Ascorbic acid	12.3	-
Doxorubicin	-	0.2

HepG2: Hepatocellular carcinoma; DPPH: 1,1-diphenyl-2-picrylhydrazyl

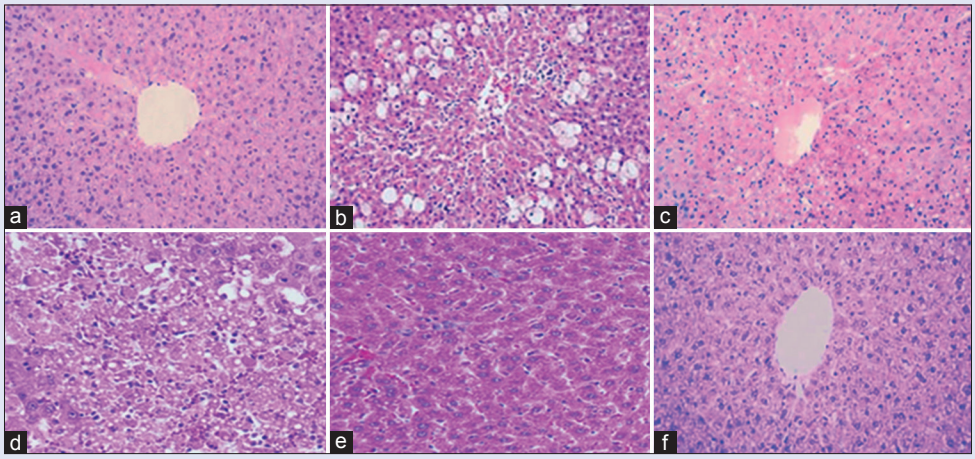

Figure 2: Histopathological appearance of rat liver cells; (a) normal cells; (b) carbon tetrachloride-treated liver cells; (c) carbon tetrachloride- and silymarin-treated liver cells; (d) carbon tetrachloride- and ethyl acetate extract (500 mg/kg)-treated liver cells; (e) carbon tetrachloride- and aqueous extract (500 mg/kg)-treated liver cells; (f) carbon tetrachloride- and total flavonoids of *Alhagi maurorum* (300 mg/kg)-treated liver cells

Table 3: Effects of the different *Alhagi maurorum* extracts on the various serum biochemical parameters in rats

Groups (*n*=6)	Dose (mg/kg)	Biochemical parameters							
		SGPT (IU/L)		SGOT (IU/L)		ALP (IU/L)		Bilirubin (mg/dL)	
		Mean±SE	Percentage reduction	Mean±SE	Percentage reduction	Mean±SE	Percentage reduction	Mean±SE	Percentage reduction
Normal saline (control)	Normal saline	41.57±3.29	-	82.41±4.65	-	267.62±5.22	-	0.45±0.04	-
CCl_4	1 ml/kg	277.86[a]±9.41***	-	349.71[a]±9.91***	-	543.25[a]±11.53***	-	3.14[a]±0.51***	-
Silymarin + CCl_4	20	64.13[b]±4.63***	76.9	111.84[b]±3.88***	68.0	275.47[b]±5.72***	49.3	0.72[b]±0.15***	77.1
Total alcohol extract + CCl_4	250	176.72[b]±6.87*	36.4	249.34[b]±6.31*	28.7	418.85[b]±8.31*	22.9	2.38[b]±0.23*	24.2
Total alcohol extract + CCl_4	500	130.31[b]±6.12**	53.1	185.02[b]±3.21**	47.1	321.55[b]±5.93*	40.8	1.77[b]±0.13*	43.5
Chloroform extract + CCl_4	250	219.61[b]±7.66	21.0	264.27[b]±6.58	24.4	441.85[b]±9.53	18.7	2.46[b]±0.38	21.6
Chloroform extract + CCl_4	500	177.33[b]±6.72*	36.2	232.75[b]±5.16*	33.4	379.76[b]±7.43*	30.1	2.01[b]±0.34*	35.9
Ethyl acetate extract + CCl_4	250	199.68[b]±7.03	28.1	248.16[b]±6.52*	30.5	404.18[b]±8.24	25.6	2.31[b]±0.42	26.4
Ethyl acetate extract + CCl_4	500	147.57[b]±5.85*	46.9	179.44[b]±4.11*	48.7	330.83[b]±6.21*	39.1	1.76[b]±0.33*	44.1
Aqueous extract + CCl_4	250	181.72[b]±4.96*	34.6	205.27[b]±5.72*	41.3	385.70[b]±7.65*	29.7	1.89[b]±0.20*	39.7
Aqueous extract + CCl_4	500	114.36[b]±4.31**	58.8	124.81[b]±4.92**	64.3	277.60[b]±7.21**	48.9	1.39[b]±0.17**	55.6
TFAM + CCl_4	150	151.54[b]±6.96*	45.5	214.37[b]±4.36*	38.7	372.05[b]±8.54*	31.5	1.84[b]±0.26*	41.4
TFAM + CCl_4	300	85.74[b]±3.66**	69.1	131.49[b]±3.87**	62.4	306.41[b]±6.55**	43.6	1.23[b]±0.11**	60.7

***P<0.001; **P<0.01; *P<0.05, [a]Compared with the control group; [b]Compared with the CCl_4 group. TFAM: Total flavonoids of *Alhagi maurorum*; SE: Standard error; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase; ALP: Alkaline phosphatase

DISCUSSION

As part of the ongoing search for the discovery of promising new hepatoprotective and antihepatoma compounds from traditional medicinal plants, we had the opportunity to thoroughly investigate the chemical constituents of *A. maurorum*. Very few reports have detailed the chemical constituents of *A. maurorum* in depth, and the majority of these studies have focused on the biological activities of the plant extract, with only a few reports discussing its chemical constituents.

In this study, 14 flavonoids (1–14) were identified from the root extract of *A. maurorum*, including four free aglycones (1–4) and ten glycoside derivatives (5–14). Compounds 6–14 were reported for the first time from *A. maurorum*. Our findings demonstrate that the isolated flavonoids possess strong radical-scavenging activity. The findings in Table 2 reveal that the antioxidant activity of the flavonoids depends on the presence of the *ortho*-dihydroxy (catechol) structure, as found in compounds 1 and 7; the presence of a 2,3-double bond and a 4-oxo function, as found in compounds 1–3; and a 3-hydroxy-4-keto and/or 5-hydroxy-4-keto conformation. Glycosylation of the free hydroxyl group at C-3 (α-hydroxyketone) may reduce the antioxidant activity. The absence of a 2,3-double bond and a 3-hydroxyl group dramatically decreases the antioxidant activity, as in compounds 4 and 14.[33]

Regarding the cytotoxic effect, all the isolated glycosides exhibited weak antiproliferative activity on the human hepatocellular carcinoma cell line HepG2 compared with the corresponding free aglycones. Moreover, the flavonols, in general, exhibited the strongest antiproliferative activity, followed by isoflavones (12, 13) and finally, the flavanones (4, 14). The strongest inhibitory effect was observed with quercetin (1), with an IC_{50} value of 18.9 μM.

The hepatoprotective activities of the different *A. maurorum* extracts and the TFAM fraction were evaluated against the hepatotoxic agent CCl_4 [Figure 2]. Numerous studies have validated that metabolism of CCl_4 in the liver results in the production of a highly reactive trichloromethyl radical that alters the cell membrane morphology and function. The modes of action of the free radicals include auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids. An elevation in the biochemical parameters such as SGPT, ALP, SGOT, and TB marks the presence of hepatic injury.

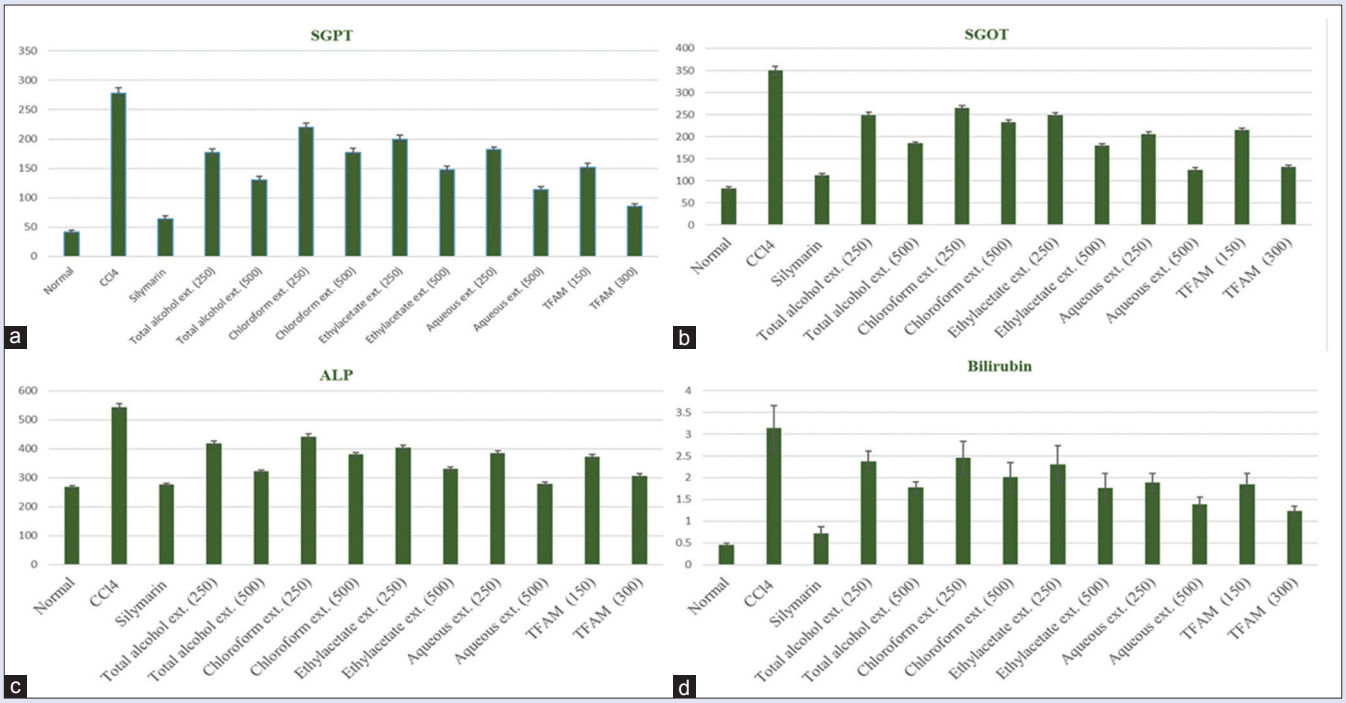

Figure 3: Effects of different *Alhagi maurorum* extracts and total flavonoids of *Alhagi maurorum* fraction on the serum biochemical parameter: (a) serum glutamate pyruvate transaminase, (b) serum glutamate oxaloacetate transaminase, (c) alkaline phosphatase, and (d) bilirubin

Table 4: Quantitative estimation of the total phenolics and flavonoids in mg/g of *Alhagi maurorum* extracts

Chemicals	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
Phenolics (mg GAE/g of dry material)	123.45±2.21	176.11±4.33	253.78±5.52
Flavonoids (mg GAE/g of dry material)	61.27±1.15	74.27±2.04	118.95±3.55

GAE: Gallic acid equivalent

The oral administration of the different *A. maurorum* extracts and the TFAM fraction reduced the biochemical parameters and exhibited hepatoprotective effects in a dose-dependent manner. The strongest hepatoprotective effect was observed with the oral preadministration of TFAM (300 mg/kg), which was still less effective than the silymarin that was used as a reference drug [Figure 3]. The mechanism of such hepatoprotective activity is likely a result of the strong inhibition of the generation of free radicals and antioxidant activity, by the flavonoids and phenolic content. Neutralizing reactive oxygen species, either by nonenzymatic mechanisms and/or enzymatic mechanisms (through enhancement of the activities of the natural hepatic-antioxidant enzymes), may be the main mechanism of TFAM against CCl_4-induced injury.[37,56] Thus, the quantitative estimation of the flavonoid content [Table 4] could explain the hepatoprotective efficiency order of the different extracts as follows: aqueous extract > ethyl acetate extract > chloroform extract.

CONCLUSION

In the current study, 14 compounds were identified from the root extract of *A. maurorum*, nine of which (6–14) were isolated for the first time. Free flavonoid aglycones, in particular, of the flavonol type, exhibited stronger antioxidant and cytotoxic activities than the flavonoid glycosides. The total flavonoid fraction (TFAM) showed the highest hepatoprotective activity, followed by the aqueous fraction, whereas the chloroform fraction exhibited the weakest effect. These data set up a scientific explanation for the traditional administration of *A. maurorum* in the treatment of general hepatic disorders. Thus, it can be concluded that *A. maurorum* is a promising hepatoprotective agent because of the antioxidant chemicals it contains.

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Conflicts of interest

There are no conflicts of interest.

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