British Journal of Pharmaceutical Research
4(16): 1963-1975, 2014

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Secondary Metabolites Isolated from Dichloromethane Fraction of Rough Lemon Stem and Hepatoprotective Effect of Limonianin

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DH and AES equally contributed. They designed the study, wrote the protocol, isolate and identified the compounds, performed the statistical analysis, wrote the first draft of the manuscript and submission. Author MW managed the analyses of the compounds, supervised the entire research and overall revision. All authors read and approved the final manuscript.

Original Research Article

Received 26th June 2014
Accepted 16th July 2014
Published 10th August 2014

ABSTRACT

Aim: To investigate the dichloromethane fraction obtained from an aqueous methanol extract of green stems of *Citrus jambhiri* Lush. and evaluate the hepatoprotective effect of limonianin.
Study Design: Isolation and identification of secondary metabolite and hepatoprotective activity of limonianin.
Place and Duration of Study: Faculty of Pharmacy, Zagazig University and Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, between February 2012 and January 2014.
Methodology: Psyllic acid, sinensetin, nobiletin, 6-demethoxytangeretin, limonianin, hesperetin, quercetin were isolated from the green stem of *Citrus jambhiri*. Their identity was unambiguously confirmed via different spectroscopic methods (UV, MS, ^{1}H NMR, ^{13}C NMR, H-COSY, HSQC and HMBC). The liver enzymes alanine aminotransferase (ALT),

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aspartate aminotransferase (AST), superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxidant capacity (TAC) were used to evaluate the hepatoprotective effect of limonianin.
Results: Limonianin exhibited an equal or even better hepatoprotective activity than the known and medicinally used flavonoid mixture silymarin on human hepatoma cells (HepG2) against D-galactosamine-induced hepatotoxicity. Limonianin at concentration 5 µM exhibited a reduction of ALT, AST and MDA with 46.50, 42.10 and 24.36%, respectively while SOD and TAC were elevated with 38.56 and 391.67%, respectively compared with D-galactosamine (D-GaIN) treated cells. At concentration of 10 µM, limonianin shows elevation of SOD and TAC with 74.56 and 791.67%, respectively compared with silymarin (63 and 766.67%, respectively).
Conclusion: The findings of this study showed that limonianin could be used as ideal hepatoprotective agent.

Keywords: Citrus jambhiri; flavonoids; limonianin; hepatoprotective.

1. INTRODUCTION

From an economical perspective trees of the genus *Citrus* (family Rutaceae) are considered to be of prime importance as they provide fruits, fragrances and medicinally useful secondary metabolites. For example, *Citrus* flavonoids exhibit antioxidant, anti-inflammatory, anti-ulcerogenic, antihepatotoxic, antidiabetic, antihypertensive, hypolipidemic, antitumor, and antimicrobial activities [1-4]. After the harvest of fruits, *Citrus* plants are regularly clipped and thereby generate high amounts of leaves and small branches. From this residual material interesting secondary products can be isolated which constitute important bioactive metabolites with potential application in pharmacology and medicine. In the framework of our detailed phytochemical and biological analysis of *Citrus jambhiri* we have isolated and analysed potential secondary metabolites with various biological activities [5-8].

So far, the green stems of *C. jambhiri* have not been phytochemically investigated. This study focuses on the isolation and structural elucidation of three polymethoxyflavones (nobiletin, sinensetin, 6-demethoxytangeretin), three flavonoid aglycones (hesperetin, quercetin, limonianin), and psyllic acid (tritriacontanoic acid). The changes associated with D-GalN-induced hepatotoxicity are similar to that of acute viral hepatitis [9]. Therefore, D-GalN-mediated hepatotoxicity was chosen as the experimental model to study the potential *in vitro* hepatoprotective activity of limonianin in comparison with standard silymarin, a known hepatoprotective flavonoid mixture from *Silybum marianum* (Asteraceae).

2. MATERIALS AND METHODS

2.1 Plant Materials

Small green stems of *Citrus jambhiri* Lush. were collected from old trees in the Research Station of the Faculty of Agriculture, Banha University, Egypt in January 2012. A voucher specimen (accession number RCJ-80-2) was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

2.2 Extraction and Isolation

The dried stem powder (2.5kg) of *C. jambhiri* was exhaustively extracted with 80% aqueous methanol (3×9L). The methanol extracts were filtered, concentrated under reduced pressure to yield 500g of a dried brownish-green residue. The residue was suspended in methanol–water (1:9) and then partitioned with light petroleum (b.p. 60–80ºC), dichloromethane and ethyl acetate successively to yield 18, 17 and 11g of final residues, respectively. About 15g of dichloromethane residue was chromatographed over a silica gel column (150 × 2.5cm, 300g). The column was eluted with light petroleum and the polarity was increased gradually using dichloromethane followed by methanol. Fractions of 250 ml each were collected, concentrated under vacuum and monitored by TLC using pre-coated Silica gel GF_{254} (Merck). Mixture of dichloromethane: acetone (9: 1 or 8.5: 1.5 v/v) or dichloromethane: methanol (9.6: 0.4 or 9: 1 v/v) were used as developing solvents. Spots were visualized with UV or by spraying with 10% aqueous H_2SO_4 followed by heating at 105ºC for 5min. Further column chromatography, crystallization and/or preparative TLC resulted in the isolation of seven compounds (1-7).

2.3 Spectral Analysis

Electron impact mass spectra (EIMS) were recorded on a MAT 8200 instrument with electron energy 70eV. In case of FAB-MS, 3-nitrobenzyl alcohol was used as a matrix. NMR spectra (^1H and ^{13}C) were recorded on a Mercury 300 and VARIAN 500 at 300 and 500 MHz for ^1H measurements and 75 and 125 MHz for ^{13}C measurements, respectively. CD_3OD, DMSO-d_6 and $CDCl_3$ were used as solvents. Chemical shits are given in ppm with TMS as internal standard. APT, 2DNMR, H-H COSY and HMPQC experiments were applied to gain reliable assignments. Experimental data were processed using MestRe-C software.

Psyllic acid (tritriacontanoic acid) (**1**): Yellowish white amorphous powder (4mg, dichloromethane/methanol); ^1H NMR ($CDCl_3$, 500 MHz): δ = 2.28 (2H, t, *J* = 7.5 Hz, H-2), 1.57 (2H, m, H-3), 1.27 (2H, m, H-32), 1.26 (6H, m, H-4, 30, 31), 1.22 (50H, m, H-5: H-29), 0.81 (3H, t, *J* = 6.95 Hz, H-33). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 178.4 (C-1), 33.8 (C-2), 31.9 (C-31), 29.8 (C-6: C-29**),** 29.3 (C-5, C-30), 29 (C-4), 24.2 (C-3), 22.2 (C-32), 15.6 (C-33) [10]. EIMS (rel. int.): 494 $[M]^+$; ($C_{33}H_{66}O_2$), 466 (1), 452 (5), 424 (9), 396 (38), 382 (52), 368 (100), 354 (28), 340 (14), 326 (10), 293 (16), 185 (11), 73 (16), 71 (19), 57 (25).

Sinensetin (**2**): Yellowish white amorphous powder (4mg, dichloromethane/ methanol); UV (MeOH) λmax 329, 264, 243 nm; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.61 (1H, dd, *J*= 9 Hz, 2 Hz, H-6'), 7.43 (1H, d, *J*= 2 Hz, H-2'), 7.00 (1H, d, *J*= 9 Hz, H-5'), 6.76 (1H, s, H-8), 6.45 (1H, s, H-3), 4.02 (3H, s), 3.99 (3H, s), 3.98 (3H, s) and 3.96 (6H, s); FAB-MS $[M^+ +1]$ at *m/z* 373; EIMS (rel. int.): 372 $[M]^+$ (100), 357 (99), 328 (20), 299 (10), 255 (1), 195 (12), 167 (48), 157 (5) [11-13].

Nobiletin **(**5,6,7,8,3′,4′-hexamethoxyflavone) (**3**): Yellowish-white needles (20mg, chloroform/ acetone); UV (MeOH) λmax 330, 270, 248 nm; ^1H NMR ($CDCl_3$, 300 MHz): δ = 7.4 (1H, d, *J*=2.1 Hz, 2′), 6.97 (1H, d, *J*=8.5 Hz, 5′) 7.55 (1H, dd, *J*=2.1, 8.5 Hz, H-6′), 6.59 (1H, s, H-3), 4.08, 4.06, 3.98, 3.93, 3.92×2 (each 3H, s, 6 OMe at C-5, -6, -7, -8, -3′, -4′). ^{13}C NMR ($CDCl_3$, 300 MHz): δ = 177.3 (s, C-4), 160.9 (s, C-2), 151.8 (s, C-4′), 151.3 (s, C-7), 149.1 (s, C-3′), 148.3 (s, C-9), 147.6 (s, C-8), 143.9 (s, C-5), 137.9 (s, C-6), 123.9 (s, C-1′), 119.5 (d, C-6′), 114.7 (s, C-10), 111.1 (d, C-5′), 108.4 (d, C-2′), 106.8 (d, C-3), 62.2, 61.9, 61.8, 61.6, 55.9, 55.8 (6 q, OMe at C-5, -6, -7, -8, -3′, -4′); FAB-MS $[M^+ +1]$ at *m/z* 403, EI/MS (rel. int.):

m/z 402 [M] $^{+}$, 387 (100), 372 (4), 357 (10), 314 (1), 197 (8) 165 (8), 162 (3), 147 (8), 83 (0.3) [12-14].

6-Demethoxytangeretin (5, 7, 8, 4'-tetramethoxyflavone) (**4**): Yellowish-white needles (3mg, dichloromethane/ methanol); UV (MeOH) λmax 344, 279, 254 nm; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.87 (2H, d, *J*= 9 Hz, H-2', 6'), 6.97 (2H, d, *J*= 9 Hz, H-3', 5'), 6.39 (1H, s, H-8), 3.29, 3.96, 3.93, 3.89, 3.83 (each 3H, s, 5 OMe); FAB-MS [M^{+}+1] at *m/z* 343, EI/MS (rel. int.): 342 [M] $^{+}$ (100), 327 (99), 298 (24), 269 (13), 255 (3), 195 (14), 157 (26), 139 (30), 89 (9) [11-13].

Limonianin (atalantoflavone) (**5**): Yellow needles (30mg, dichloromethane/ methanol); UV (MeOH) λmax 327, 310, 273, 231 nm; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 7.92 (1H, d, *J*=9.6 Hz, H-2', 6'), 6.93 (2H, d, *J*= 9.6 Hz, H-3', 5'), 6.90, 1H, d, *J*=10 Hz, H-1´´), 6.85 (1H, s, H-3), 6.21 (1H, s, H-6), 5.81 (1H, d, J=10 Hz, H-2´´), 1.4 (6H, s, *gem* dimethyl); ^{13}C NMR (DMSO-d_6, 300 MHz): δ = 182.7 (C-4), 164.5 (C-2), 162.1 (C-4′), 161.6 (C-5),, 159.6 (C-7), 151.9 (C-9), 129.3 (C-6′& 2′), 128.8 (C-2´´), 121.7 (C-1′), 116.8 (C-3´&5´), 115.1 (C-1´´), 105.3 (C-10), 103.6 (C-3), 101.7 (C-8), 100.0 (C-6), 78.8 (C-3´´), 28.4 (*gem* dimethyl); ESI-MS [M^{+}+1] at *m/z* 337, EI/MS (rel. int.): 336 [M] $^{+}$ (33), 322 (26), 321 [M-Me] $^{+}$ (100), 293 [M-MeCO] $^{+}$ (1), 203 (RDA of [M-Me] $^{+}$) (32), 161 (2), 118 (3), 77 (2), 69 (2) [15-17].

Hesperetin (**6**): Dark yellow amorphous powder (5mg, dichloromethane/ methanol); UV (MeOH) λmax 288; ^{1}H NMR (CD_3OD, 300 MHz): δ = 6.99 (1H, d, *J*= 2.1 Hz, H-2'), 6.91 (1H, dd, *J*= 8.7, 2.1 Hz, H-6'), 6.86 (1H, d, *J*= 8.7Hz, H-5'), 5.90 (1H, d, *J*=2 Hz, H-8), 5.85 (1H, d, *J*=2 Hz, H-6), 5.5 (1H, dd, *J*=7.6, 3.1 Hz, H-2), 3.83 (3H, s, OCH_3), 3.09 (1H, m, H-3α), 2.65 (1H, m, H-3β). ^{13}C NMR (CD_3OD, 300 MHz): δ = 196.4 (C-4), 167.3 (C-7), 164.3 (C-5), 163.6 (C-9), 148.2 (C-4′), 146.6 (C-3′), 131.9 (C-1′), 117.8 (C-6′), 113.3 (C-2′), 111.4 (C-5′), 102.2 (C-10), 95.9 (C-6), 95.0 (C-8), 79.1 (C-2), 56.1 (C-4′, OCH_3); EI/MS (rel. int.): at m/z 302 (100), 259 (6.99), 153 (16), 150 (22), 137 (31), 135 (16), 124 (7), 111 (1), 107 (6), 78 (20), 77 (24) [18].

Quercetin (**7**): Yellow granules (8mg, dichloromethane/ methanol); UV (MeOH) λmax 372, 301(sh), 269 (sh), 255 nm; ^{1}H NMR (CD_3OD, 300 MHz): δ = 6.88 (1H, d, *J*= 2.1 Hz, H-2'), 7.73 (1H, dd, *J*= 8.7, 2.2 Hz, H-6'), 7.61 (1H, d, *J*= 8.7 Hz, H-5'), 6.38 (1H, H-8), 6.18 (1H, H-6); (CD_3OD, 300 MHz): δ = 176.2 (C-4), 165.1 (C-7), 161.9 (C-5), 156.9 (C-9), 147.9 (C-2), 147.5 (C-4′), 145.9 (C-3′), 136.1 (C-3), 123.8 (C-1′), 121.2 (C-6′), 115.9 (C-5′), 115.2 (C-2′), 103.9 (C-10), 98.1 (C-6), 93.9 (C-8); EI/MS (rel. int.): at m/z 302 (100), 274 (11), 245 (9), 153 (14), 150 (4), 147 (5), 137 (26), 128 (9), 121 (11), 109 (15), 81 (30), 69 (34) [18].

2.4 Chemicals

Chemicals were purchased from Sigma Aldrich GmbH (Sternheim, Germany), AppliChem (Darmstadt, Germany) and Fluka (Buchs, Switzerland). D-galactosamine and silymarin were supplied by Sigma Chemicals (ST. Louis. Mo, USA). All solvents for extraction and separation were of analytical grade. Cell culture reagents were obtained from Lonza (Basel, Switzerland).

2.5 Cell Culture

Hepatotoxicity of D-GalN can be determined in HepG2 human hepatoma cells. This cell line was first purchased from VACSERA, Giza, Egypt, and then maintained under standard conditions. Cells were grown in RPMI medium supplemented with heat-inactivated fetal

bovine serum (10 %), penicillin G (100 IU/mL) and streptomycin (100 µg/mL). Cells were maintained at 37ºC in a 5% CO_2 atmosphere with 95% humidity.

2.6 Hepatoprotective Activity

The hepatoprotective activity of limonianin (purity > 97%) was tested *in vitro* in two different concentrations (5 and 10 µM) and compared to 10 µM silymarin (dissolved in DMSO). The HepG2 monolayer culture was pretreated with the flavonoids for 1 h. Then 10 µM D-GalN was added and incubation was continued for another 2 h. The supernatant medium and cell lysate were then collected and stored at −20ºC until analysis. As a positive control cells were treated only with10 µM D-GalN, while the blank control was a set of cells maintained in phosphate-buffered saline. The levels of the enzymes ALT, AST (U/L) in the media were evaluated according to Reitman and Frankel [19]. Parameters of oxidative stress profile were assayed by colorimetric technique using commercial kits (Biodiagnostic, Egypt). Total antioxidant capacity (TAC) (mmol/L) and superoxide dismutase (SOD) (U/mL) were determined in cell lysate [20, 21]. Lipid peroxidation content was evaluated by measurement of MDA (nmol/mL) [22].

2.7 Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA followed by Tukey post hoc test using a computer-based curve fitting program (prism 5, Graphpad,CA, USA). *P* value = 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Investigation

Column chromatography of the dichloromethane fraction of green stems of *C. jambhiri* lead to the isolation of seven major compounds (**1-7**) (Fig. 1). The structures of these compounds were unambiguously determined by their chromatographic behaviour as well as spectroscopic methods, including 1D and 2D NMR experiments and MS analysis with regard to published data. To our knowledge, the isolated and identified compounds have not been reported before from the stem of *C. jambhiri*. However, nobiletin and 6-demethoxytangeretin were previously reported as components in the peel of this plant [6,23,24].

Banerji et al. [15] reported the isolation of a flavonoid aglycone atalantoflavone from the leaves of *Atalantia racemosa* with its ^{1}H-NMR. Two years later, ^{13}C-NMR of a similar compound (named limonianin) from the root bark of *Citrus limonia* had been reported [16] but the interpretation of ^{13}C-NMR data are not satisfactory to deduce the structure. Thus, we have carried out a reassignment of this compound **5** with the aid of HCOSY, HSQC and HMBC experiments. The ^{13}C-NMR values recorded for limonianin (**5**) differ from those already reported [16] and suggest that the compound isolated from the root bark of *C. limonia* was a stereoisomer of the metabolite described here. The unambiguous assignment of NMR signals of **5** was achieved on the basis of one-dimensional (1D) and two-dimensional (2D) NMR techniques such as HCOSY, HMQC, HSQC and HMBC. A pair of coupled doublets at δ_H 7.92 and 6.93 (each 2H, *J*=6.9Hz, H-2'/H-6', H-3'/H-5') and their carbons δ_C 129.30 (each CH, C-2'/6'), 116.76 (each CH, C-3'/C-5') showed the presence of a *para* substituted ring B. The presence of hydroxyl group at C-4' was established from the mass fragment at *m/z* 118 in EIMS. The fragment ion at *m/z* 203 which corresponds to an

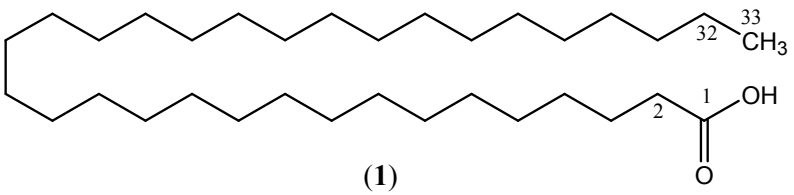
[ring A^++H] fragment after retro Diels-Alder cleavage such as a flavonoid compound suggested that the dimethylchromone moiety was substituted on the A ring [25]. A singlet at δ 6.85 is assigned to the proton at position 3 (δ_C 103.64). In the ^{1}H-NMR spectrum, the *gem*-dimethylchromene ring was observed as one 6H singlet at δ 1.40 and two 1H doublets due to olefinic protons at δ 6.9 (1H, d, *J*=10Hz) and δ 5.81 (1H, d, *J*=10 Hz). An hydroxyl group at C-5 was deduced from the NMR spectrum resonated at δ 13.6 as a broad singlet. This clearly indicated that the *gem*-dimethylchromone moiety was fused to C-7 and C-8 [17,25,26]; this placement was supported by correlation in the HMBC spectrum. On the basis of these results the structure of **5** is concluded to be limonianin [15-17].

3.2 Hepatoprotective Effect of Limonianin in Cultured Human Liver Cells HepG2

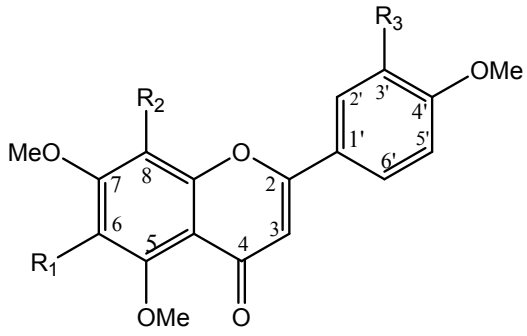
Great attention has been paid to the protective effects of natural antioxidants such as silymarin against drug-induced toxicity studies especially whenever free radical generation is involved [27,28]. An hepatoprotective activity of flavonoids is well-known as it plays a very important role in protection against oxidative stress [29-31]. Sinensetin [32-34], nobiletin [35-37], quercetin [38,39] and hesperetin [40] were previously investigated for their hepatoprotective activity. Limonianin was selected in this study to evaluate its *in vitro* hepatoprotective activity in HepG2 cells against D-galactosamine-induced hepatotoxicity. The levels of hepatic damage markers such as aspartate transaminase (AST) and alanine transaminase (ALT) after treatment with D-galactosamine were significantly increased by 517 and 244%, respectively, compared to the control. Oxidative stress markers such as the activities of superoxide dismutase (SOD) and total antioxidant capacity (TAC) were significantly decreased (48% and 70%, respectively) and the lipid peroxidation product (MDA) was enhanced (104%).

D-galactosamine-induced increase in ALT and AST activities were significantly suppressed by treatment of HepG2 cells with 5 and 10 µM limonianin (Figs. 2,3**)**. The level of both TAC (392%) and the activities of the antioxidative enzyme SOD (39%) were significantly replenished by pretreatment of the same cells with 5 µM as compared to cells treated with D-galactosamine alone (Figs. 4,5). In addition, MDA level (24%) was reduced by the same doses of limonianin compared to cells treated with D-galactosamine (Fig. 6). Pre-treatment of HepG2 cells with 10 µM limonianin resulted in better hepatoprotective values than the silymarin under the same conditions. D-galactosamine appears to cause liver injury by inhibiting the synthesis of RNA and proteins [41-44].

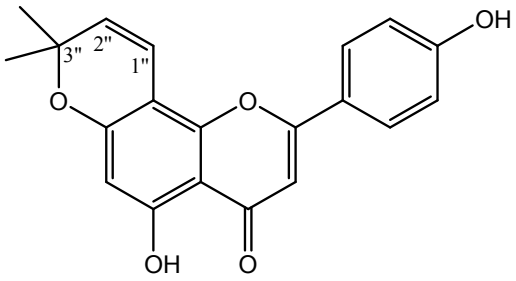
D-GalN is known to increase the membrane permeability of cultured human hepatocytes as confirmed by marked increase of both ALT and AST seen in our experiments. This may further increase membrane lipid peroxidation which would enhance MDA levels. The antioxidant property of limonianin is apparent from the significant increase of TAC and SOD.



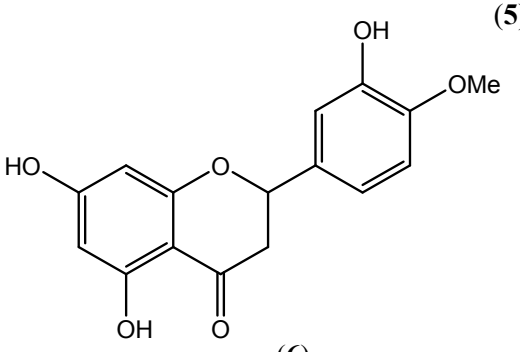
(**1**)



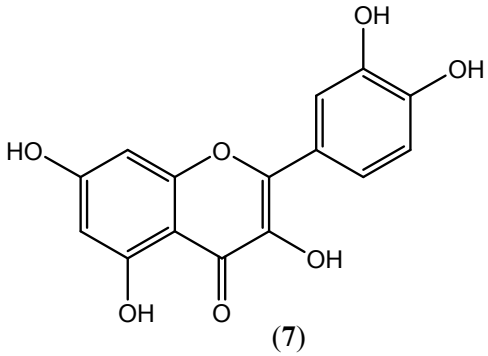
R_1	R_2	R_3	
OMe	H	OMe	(**2**)
OMe	OMe	OMe	(**3**)
H	OMe	H	(**4**)



(**5**)



(**6**)



(**7**)

Fig. 1. Isolated compounds from green stems of *C. jambhiri*

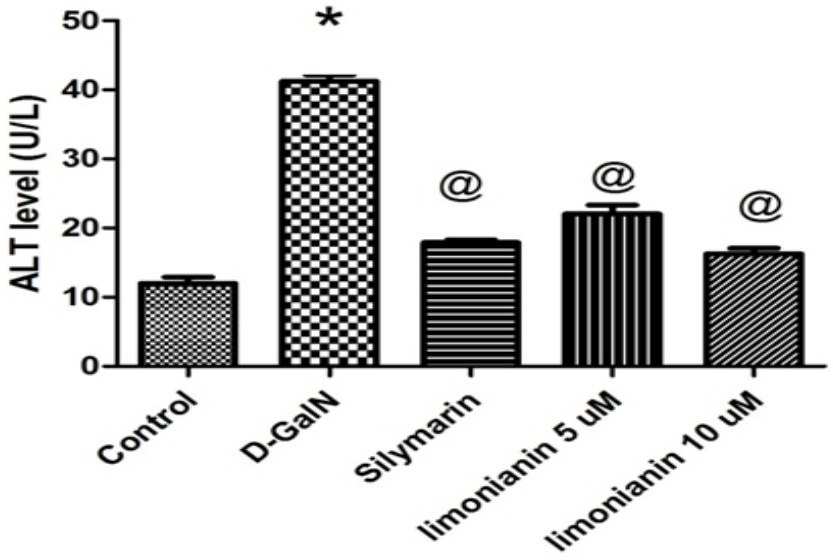


Fig. 2. Effect of limonianin (5 and 10μM) and silymarin (10 μM) on liver enzyme ALT in D-GalN-treated HepG2cells (in media), Data represent means of three experiments±SEM

** Significant difference compared to control, (@) significant difference compared to D-GalN-treated cells, P = 0.05*

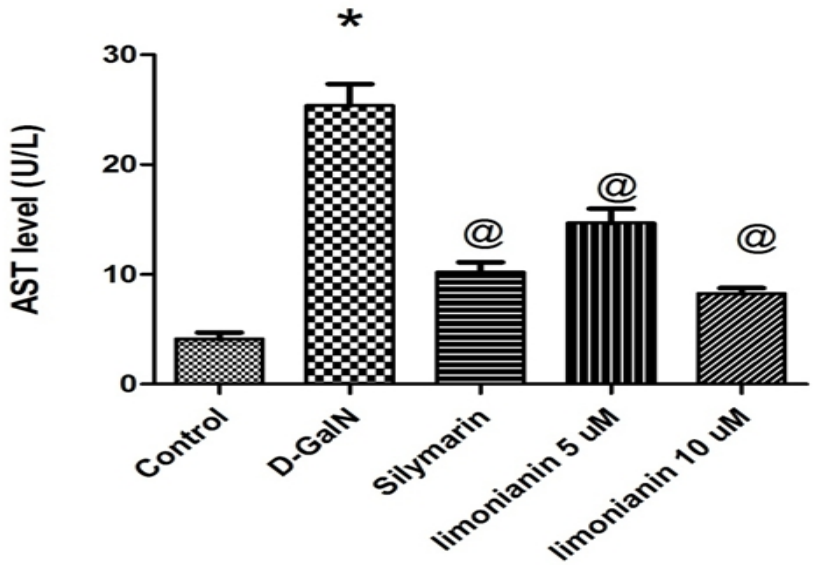


Fig. 3. Effect of limonianin (5 and 10μM) and silymarin (10 μM) on liver enzyme AST in D-GalN-treated HepG2 cells (in media), Data represent means of three experiments ± SEM

** Significant difference compared to control, (@) significant difference compared to D-GalN-treated cells, P = 0.05*

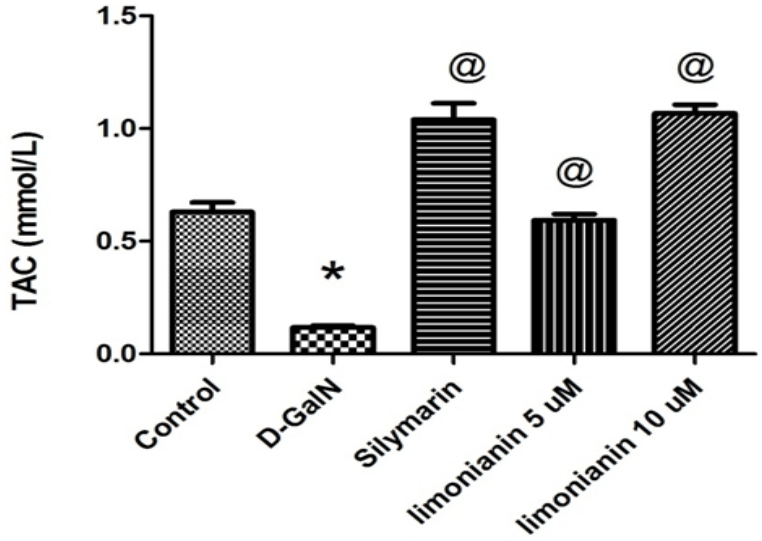


Fig. 4. Effect of limonianin (5 and 10μM) and silymarin (10 μM) on total antioxidant capacity (TAC) in D-GalN-treated in HepG2 cells (in cell lysate), Data represent means of three experiments ± SEM

** Significant difference compared to control, (@) significant difference compared to D-GalN-treated cells, P = 0.05*

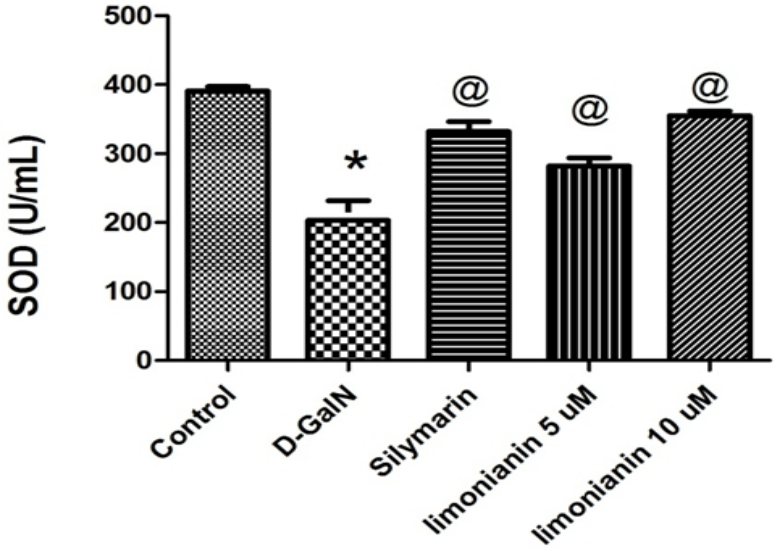


Fig. 5. Effect of limonianin (5 and 10μM) and silymarin (10 μM) on superoxide dismutase (SOD) in D-GalN-treated HepG2 cells (in cell lysate), Data represent means of three experiments ± SEM

** Significant difference compared to control, (@) significant difference compared to D-GalN-treated cells, P = 0.05*

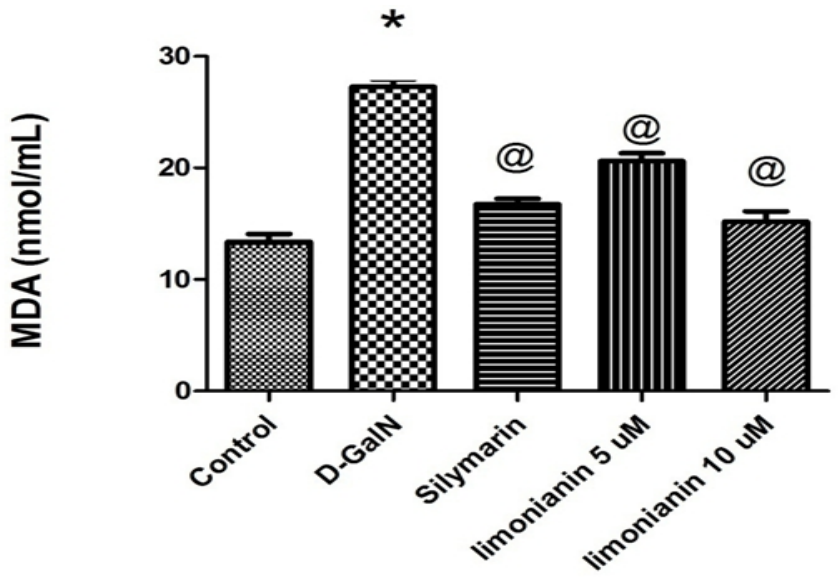


Fig. 6. Effect of limonianin (5 and 10μM) and silymarin (10μM) on malondialdehyde (MDA) in D-GalN-treated in HepG2 cells (in cell lysate), Data represent means of three experiments ± SEM

** Significant difference compared to control, (@) significant difference compared to D-GalN-treated cells, P = 0.05*

4. CONCLUSION

Limonianin isolated from green stems of *Citrus jambhiri* exhibits pronounced hepatoprotective properties that are equal or even better than those of silymarin. Thus, limonianin should have a great potential for the treatment of liver hepatotoxicity which needs to be confirmed under *in vivo* conditions.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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