Digalacturonide Flavones from Egyptian *Lantana camara* Flowers with *in vitro* Antioxidant and *in vivo* Hepatoprotective Activities

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A new digalacturonide flavone, luteolin 7-*O*-*β*-galacturonyl-(2→1)-*O*-*β*-galacturonide (**1**), was isolated along with nine known flavone glycosides from the aqueous methanolic extract of *Lantana camara* (L.) flowers. Their structures were determined on the basis of the spectral data. The extract of *L. camara* was evaluated for antioxidant and hepatoprotective properties in the acetaminophen-induced mouse liver damage model. **1** exhibited significant antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay with an IC_{50} value of 27.2 μM. Pre-treatment with *L. camara* extract (25 and 75 mg/kg body weight) decreased the activities of alkaline phosphatase (ALP), serum glutamate oxaloacetate transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT) enzyme levels that were elevated by acetaminophen. Both doses of the *L. camara* extract ameliorated the histopathological and histochemical alterations induced by acetaminophen. The results indicate that the *L. camara* extract possesses hepatoprotective activity against acetaminophen-induced liver damage.

Key words: Digalacturonide, *Lantana camara*, Acetaminophen

Introduction

Lantana camara L. (Verbenaceae), commonly known as wild or red sage, is the most widespread species of this genus, growing luxuriantly at elevations up to 2000 m in tropical, sub-tropical, and temperate regions (Sharma *et al.*, 1988). It is a woody straggling plant with various flower colours, red, pink, white, yellow, and violet.

It is used in folk medicine as vulnerary, diaphoretic, carminative, antiseptic, antispasmodic tonic, appetizer, and vomitive. Various parts of the plant are used in the treatment of itches, cuts, ulcers, swellings, bilious fever, catarrh, eczema, dysentery, chest complaints of children, fistula, pustules, tumours, tetanus, malaria, rheumatism, toothache, cold, headache, uterine haemorrhage, chicken pox, eye injuries, whooping cough, asthma (Chopra *et al.*, 1956; Anonymous, 1962; Agarwal, 1997), bronchitis, and arterial hypertension (Chopra *et al.*, 1969; Rastogi and Mehrotra, 1995). It has been recorded that different parts of *L. camara* are a rich source of various bioactive principles such as triterpenes of the lantadene type, *e.g.* lantadenes A and B, lantic acid, and lantanolic acid (Barre *et al.*, 1997). The presence of iridoide glycosides, oligosaccharides, phenylpropanoid glycosides, and naphthoquinones has been reported. A number of flavonoid compounds have been isolated from *L. camara.* 3-Methoxy-3,7-dimethoxy- and 3,7,4'-trimethoxyquercetin, respectively, were isolated from the leaves of *L. camara* whereas hispidulin was isolated from the stems. The flavone glycoside camaraside has also been isolated from *L. camara* var. *aculeata* together with pectolinarigenin 7-*O*-*β*-glucoside (Sharma *et al.*, 2007).

Some taxa of *L. camara* are toxic to ruminants where several cases have been reported from Australia, India, New Zealand, South Africa, and the Americas (Seawright *et al.*, 1983; Pass, 1991). The triterpene fraction containing the toxic lan-

tadenes can readily be separated from the more polar components, *i.e.* the phenylethanoids, iridoid glycosides, and flavonoids, by solvent fractionation (Sharma *et al.*, 2007).

This work deals with the isolation and structure elucidation of a new digalacturonide flavone along with nine known flavone glycosides from *L. camara* flowers. The antioxidant activity of the extract and the isolated compounds was evaluated by checking their scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The extract was evaluated for hepatoprotective and curative activity against acetaminophen-induced liver injury in mice. Histological examination was also performed and correlated to the biochemical parameters.

Results and Discussion

Characterization of the isolated compounds

The aqueous methanolic (70%) extract of *L. camara* flowers was fractionated with *n*-hexane and dichloromethane. The aqueous fraction was subjected to polyamide column chromatography to afford flavones **1**–**10** as a pale yellow, amorphous powder. All compounds appeared as dark purple spots on paper chromatograms under UV light, changing to yellow when exposed to ammonia vapour, except compound **7** for which no change in colour was observed. The UV data confirmed that these compounds contained apigenin and luteolin, respectively (Mabry *et al.*, 1970).

Based on chromatographic and spectroscopic analyses, the isolated compounds were identified as luteolin 7-*O*-*β*-galacturonyl-(2→1)-*O*-*β*-galacturonide (**1**), apigenin 7-*O*-*β*-galacturonyl-(2→1)-*O*-*β*-galacturonide (**2**), luteolin 7-*O*-*β*-glucuronyl-(2→1)-*O*-*β*-glucuronide (**3**), apigenin 7-*O*-*β*-galacturonide (**4**), luteolin 7-*O*-*β*-glucoside (**5**), luteolin 7-*O*-*β*-galactoside (**6**), luteolin 4'-*O*-*β*-glucoside (**7**), apigenin 7-*O*-*β*-glucoside (**8**), vitexin (**9**), and isovitexin (**10**). Complete acid hydrolysis of compounds **1**–**8** yielded luteolin and apigenin, respectively (UV and ^{1}H NMR), as the flavone components together with galacturonic acid, glucuronic acid, glucose, and galactose as sugar moieties. The components were co-chromatographed with the authentic samples (Mabry *et al.*, 1970). The sugar moieties were also detected by subjecting the aqueous layer of the hydrolysis solutions to high-performance liquid chromatography (HPLC) using a refractive index detector. The hydrolysable compounds **1**–**4** and standard samples of glucuronic and galacturonic acids were injected. The sugars of compounds **1**, **2**, and **4** were detected at R_t = 6.7 min matching well with authentic galacturonic acid, while the sugar of compound **3** was detected at R_t = 6.1 min matching well with authentic glucuronic acid.

UV spectra in MeOH of **1** gave absorption maxima at 351 (band I) and 255 nm (band II) which are characteristic for flavones. A bathochromic shift with NaOMe without decrease in intensity of band I (402 nm) was diagnostic for the presence of a free 4'-OH group. The lack of a bathochromic shift of band II (266 nm) in the presence of NaOAc relative to the same band in MeOH suggested that the 7-hydroxy group is substituted (Mabry *et al.*, 1970). ESI mass spectrometry of **1** gave the molecular ion peak at *m/z* 639 $[M+H]^+$ suggesting that it has two galacturonic acid moieties.

The ^{1}H NMR spectrum of **1** (Table I) revealed resonances consistent with the presence of two galacturonic acid moieties and luteolin. The spectrum showed the proton signals of luteolin as a chelated hydroxy signal of 5-OH with 4-keto at δ_H 12.83 ppm; three doublets of ring B at δ_H 7.41 (H-2′), 7.39 (H-6′), and 6.88 ppm (H-5′); two *meta*-doublets at δ_H 6.79 and 6.37 ppm assigned to protons H-8 and H-6, respectively. The glycosidic nature of **1** was confirmed from the appearance of two anomeric proton doublets at δ_H 5.07 and 4.52 ppm with the coupling constant 7.5 Hz. The high *J* values indicated the *β*-configuration of the two galacturonic acids (Markham and Geiger, 1994).

The decoupled ^{13}C NMR spectrum of **1** (Table I) displayed resonance of the typical 15 luteolin carbon signals and 12 carbon signals for the two galacturonic acid moieties. In the spectrum, the characteristic carbon signals of the two galacturonic acid moieties appeared at δ_C 99.8 (C-1″) and 103.7 ppm (C-1‴), while the downfield carboxylic carbon signals of C-6″ and C-6‴ appeared at δ_C 170.7 and 170.4 ppm, respectively. The downfield shift of the galacturonic acid C-2'' signal (δ_C 82.2 ppm) suggested that the interglucosidic linkage was (1→2).

The HMBC experiment of **1** (Fig. 1) was set at the delay time of 50 ms, corresponding to a *J*(C/H) value of 8 Hz. The HMBC spectrum (Table II) showed all expected $J_{C,H}$ correlations and permitted unambiguous assignment of all reso-

Table I. ^{1}H (500 MHz) and ^{13}C NMR (125.7 MHz) data of compounds **1**, **2**, and **4** in DMSO-d_6 with shifts as δ values, coupling constants in Hz.

C/H	**1**		**2**		**4**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	-	165.0	-	164.9	-	164.8
3	6.77 s	104.8	6.80 s	103.6	6.81 s	103.6
4	-	182.5	-	182.5	-	182.5
5	-	157.4	-	157.2	-	157.5
6	6.37 d, 2.3	98.1	6.61 d, 2.1	98.6	6.45 d, 1.9	100.0
7	-	162.7	-	163.4	-	163.4
8	6.79 d, 2.3	95.1	7.12 d, 2.1	96.2	6.81 d, 1.9	95.2
9	-	161.7	-	163.1	-	161.9
10	-	106.0	-	105.9	-	105.8
1’	-	121.9	-	120.4	-	121.5
2’	7.41 d, 2.3	114.1	7.92 d, 8.2	128.9	7.90 d, 8.4	129.2
3’	-	146.3	6.88 d, 8.2	116.7	6.89 d, 8.4	116.6
4’	-	150.4	-	161.3	-	161.6
5’	6.88 d, 8.5	116.5	6.88 d, 8.4	116.7	6.89 d, 8.4	116.7
6’	7.39 d, 8.5	119.7	7.92 d, 8.4	128.9	7.91 d, 8.4	129.2
1’’	5.07 d, 7.5	99.8	5.05 d, 7.5	100.9	5.08 d, 7.6	100.1
2’’	3.62 dd, 9.0	82.8	3.68 d, 9.0	81.7	3.26 d, 9.0	72.2
3’’	3.42 – 3.24 br	75.4	3.35 – 3.15 br	74.4	3.66 – 3.28 br	73.4
4’’	3.42 – 3.24 br	71.3	3.35 – 3.15 br	72.1	3.66 – 3.28 br	69.9
5’’	3.44 d, 9.0	76.1	3.41 d, 9.0	77.0	3.71 d, 9.0	76.6
6’’-COOH	-	170.7	-	173.2	-	171.7
1’’’	4.52 d, 7.5	103.7	4.43 d, 7.6	102.9		
2’’’	3.22 d, 9.0	74.6	3.68 d, 9.0	74.0		
3’’’	3.42 – 3.24 br	75.5	3.35 – 3.15 br	74.7		
4’’’	3.42 – 3.24 br	72.2	3.35 – 3.15 br	72.5		
5’’’	3.52 d, 9	76.3	3.41 d, 9.0	76.1		
6’’’-COOH	-	170.4	-	172.4		

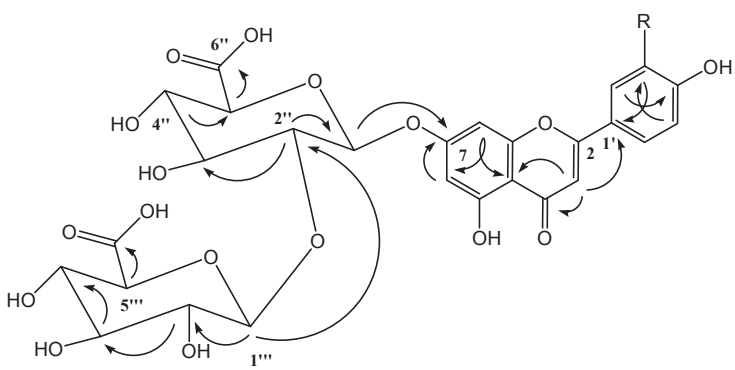

Fig. 1. Significant HMBC correlations of compounds **1** (R = OH) and **2** (R = H).

nances. The anomeric proton of galacturonic acid (δ_H 5.07 ppm) showed a strong cross-peak with δ_C 162.7 ppm (C-7) and weak correlation (^{2}J) with δ_C 82.8 ppm (C-2’’), while the anomeric proton of the second galacturonic acid moiety (δ_H 4.52 ppm) showed a strong cross-peak (^{3}J) with δ_C 82.8 ppm (C-2’’) confirming the glycosidic linkage (1→2) of the digalacturonide moiety. Consequently, the structure of compound **1** was established as luteolin 7-*O*-*β*-galacturonyl-(2→1)-*O*-*β*-galacturonide

Table II. Key HMBC correlations of **1** and **2**.

Proton	**1**		Proton	**2**	
	2*J*	3*J*		2*J*	3*J*
H-3	C-2, C-4	C-1', C-10	H-3	C-2, C-4	C-1', C-10
H-6	C-5, C-7	C-8, C-10	H-6	C-5, C-7	C-8, C-10
H-8	C-7, C-9	C-6, C-10	H-8	C-7, C-9	C-6, C-10
H-2'/6'	C-1', C-3', C-5'	C-2, C-4'	H-2'/6'	C-1', C-3'/5'	C-2, C-4'
H-5'	C-4', C-6'	C-1', C-3'	H-3'/5'	C-4'	C-1', C-2'/6'
H-1''	C-2'	C-7	H-1''	-	C-7
H-2''	C-1''	C-1'''	H-2''	C-1''	C-1'''
H-1'''	-	C-2''	H-1'''	C-2'''	C-2''

which has not been reported previously to occur in nature.

Compound **2** was slightly less polar than compound **1**, with the NMR patterns of an apigenin aglycon and a digalacturonide moiety. The structure elucidation of compound **2** was established by UV, 1D- and 2D-NMR spectroscopy along with chemical hydrolysis. Compound **2** was identified as apigenin 7-*O*-*β*-galacturonyl-(2→1)-*O*-*β*-galacturonide which has been derived from safe food substances GRAS (rosemary, sage and peppermint plant material) (Lenoble *et al*., 1999).

Antioxidant activity

The antioxidant activity of the aqueous methanolic extract of *L. camara* and the isolated pure flavone glycosides was studied *in vitro* using the DPPH method. Luteolin glycosides in the aqueous methanolic extract of *L. camara* are probably involved in the extract's radical scavenging activity (Table III). The new compound **1** and luteolin 4'-*O*-*β*-glucoside (**7**) displayed high scavenging activity against the DPPH free radical with IC_{50} values of 27.2 and 27.0 μM, respectively. The aqueous methanolic extract of *L. camara* showed weak scavenging activity against the DPPH free radical (IC_{50} 74.3 μg/ml), and compounds **2**, **4**, and **8**–**10** were inactive.

Table III. DPPH scavenging activity of the *L. camara* extract and isolated pure compounds.

Sample	IC_{50} [μg/ml]	IC_{50} [μM]
L. camara extract	74.3	--
1	17.3	27.2
3	66.3	103.9
5	52.0	116.1
6	24.3	54.2
7	12.1	27.0
Ascorbic acid	4.7	26.3

Biochemical study

Administration of acetaminophen to mice induced hepatotoxicity, which was demonstrated by a marked elevation in the activities of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) in comparison with the control group ($P < 0.05$) (Table IV).

Hepatotoxicity of drugs may be due *e.g.* to the formation of reactive metabolites, antioxidant depletion, and protein alkylation (Amin and Hamza, 2005; Pugh *et al*., 2009). For acetaminophen, the toxicity mainly depends on the expression of gene variants (CAR-dependent genes) (Zhang *et al*., 2002), and hepatocyte death follows an apoptotic or necrotic pathway mainly depending on predisposing factors (Farber, 1994; Malhi *et al*., 2006).

Pre-treatment or post-treatment of mice with 25 and 75 mg/kg body weight (BW) of the *L. camara* extract significantly ($P < 0.05$) lowered the elevations in the serum SGOT, SGPT, and ALP enzyme activities that were induced by acetaminophen (Table IV). This effect may be due to plasma membrane stabilization, thereby preserving the structural integrity of cells, as well as repairing the hepatic tissue damage caused by acetaminophen (Sherlock and Dooley, 2002; Pari and Murugan, 2004).

Histopathological study

The histology of the liver sections of control animals exhibited normal liver architecture with well-preserved cytoplasm, prominent nucleus, and hepatocytes radially arranged around the central

Table IV. Effect of the *L. camara* extract on liver enzyme activities in mice intoxicated with acetaminophen.

Group	SGOT [IU/l]	SGPT [IU/l]	ALP [IU/l]
Control	132.4 ± 9.8	88.6 ± 17.2	68.6 ± 8.9
Acetaminophen	348.6 ± 11.4*	275 ± 14.6*	172.3 ± 6.4*
L. camara extract (25 mg/kg BW) and acetaminophen	188.2 ± 3.68**	164.6 ± 13.2**	134.6 ± 3.65**
L. camara extract (75 mg/kg BW) and acetaminophen	144.5 ± 3.74**	112.4 ± 9.1**	96.8 ± 3.2**
Acetaminophen and *L. camara* extract (25 mg/kg BW)	201.1 ± 1.41**	170.2 ± 9.0**	144.3 ± 6.05**
Acetaminophen and *L. camara* extract (75 mg/kg BW)	160.5 ± 2.43**	115.5 ± 6.2**	100.2 ± 7.3**
Silymarin (50 mg/kg BW) and acetaminophen	140.21 ± 2.16**	100.45 ± 3.21**	84.44 ± 1.65**
Acetaminophen and silymarin (50 mg/kg BW)	135.3 ± 1.25**	95.91 ± 2.45**	77.61 ± 2.1**

Values are presented as means ± S.E., $n = 6$ mice per group.
* Significant different at $P < 0.05$ as compared with group I (control).
** Significant different at $P < 0.05$ as compared with group II (acetaminophen).

vein (Fig. 2A). The administration of acetaminophen induced fatty and hydropic degeneration, karyopyknosis, necrosis, loss of cellular boundaries, and inflammatory cells in the portal and periportal areas that was associated with congestion of the portal vein and blood sinusoids as shown in Fig. 2B. Acetaminophen is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats, and mice at toxic doses (Mitchell *et al.*, 1973; Kuma and Rex, 1991; Eriksson *et al.*, 1992).

The hepatohistological changes induced by acetaminophen were markedly ameliorated by the pre-treatment or post-treatment with *L. camara* extract and silymarin (Fraschini *et al.*, 2002), which significantly reduced hydropic degeneration and markedly decreased the necrosis in the hepatocytes (Figs. 2C, D, E, F, G, H).

Histochemical study

Hepatic glycogen is an important reserve of glucose for sustaining endogenous glucose production during fasting. Liver tissue of mice in the control group gave a positive periodic acid Schiff (PAS) reaction due to normal glycogen contents. The liver parenchyma showed pink granules. PAS-positive polysaccharide granules were found in the cytoplasm of most hepatocytes as intense coarse granules displaced to one pole of the cell during the fixation period (glycogen migration phenomenon) (Bancroft, 1975) (Fig. 3A). In the acetaminophen group, the liver tissues showed severe glycogen depletion. Most of the hepatocytes had lost most of their glycogen and some showed a negative PAS-reaction (Fig. 3B).

In all other mice, having received acetaminophen and pre- or post-treatment with 25 and 75 mg/kg BW of *L. camara* extract or silymarin (50 mg/kg BW), the hepatic tissue was moderately stained by PAS. Their cytoplasm appeared to be filled with a considerable amount of reddish granules, more than in the acetaminophen group (Figs. 3C, D, E, F, G, H).

In the current study, severe depletion of hepatic glycogen in the acetaminophen-treated group was observed. A considerable decrease in liver glycogen after acetaminophen treatment has been described for hepatocytes (Evdokimova *et al.*, 2001). To explain the decline in the glycogen levels, Burcham and Harman (1989) speculated that the decline might be mediated through ionic disturbances, especially loss of calcium homeostasis, which may lead to the activation of glycogen phosphorylase.

The present study clearly demonstrates that the *L. camara* extract possesses hepatoprotective and curative activities against acetaminophen-induced liver damage as indicated by the biochemical, histopathological, and histochemical investigations.

Material and Methods

Materials

HPLC analysis was performed on Shimadzu SCL-10Avp instrument (Kyoto, Japan) equipped with a column (C18; Shodex, Kyoto, Japan), refractive index (RId-10A) detector, pump (LC-10ADvp), and degasser (DGU-14A). UV spectra were obtained using Beckman DU7 (Tokyo, Japan) and Shimadzu UV 240 (PIN 204–5800) spectrometers (Tokyo, Japan). NMR experiments

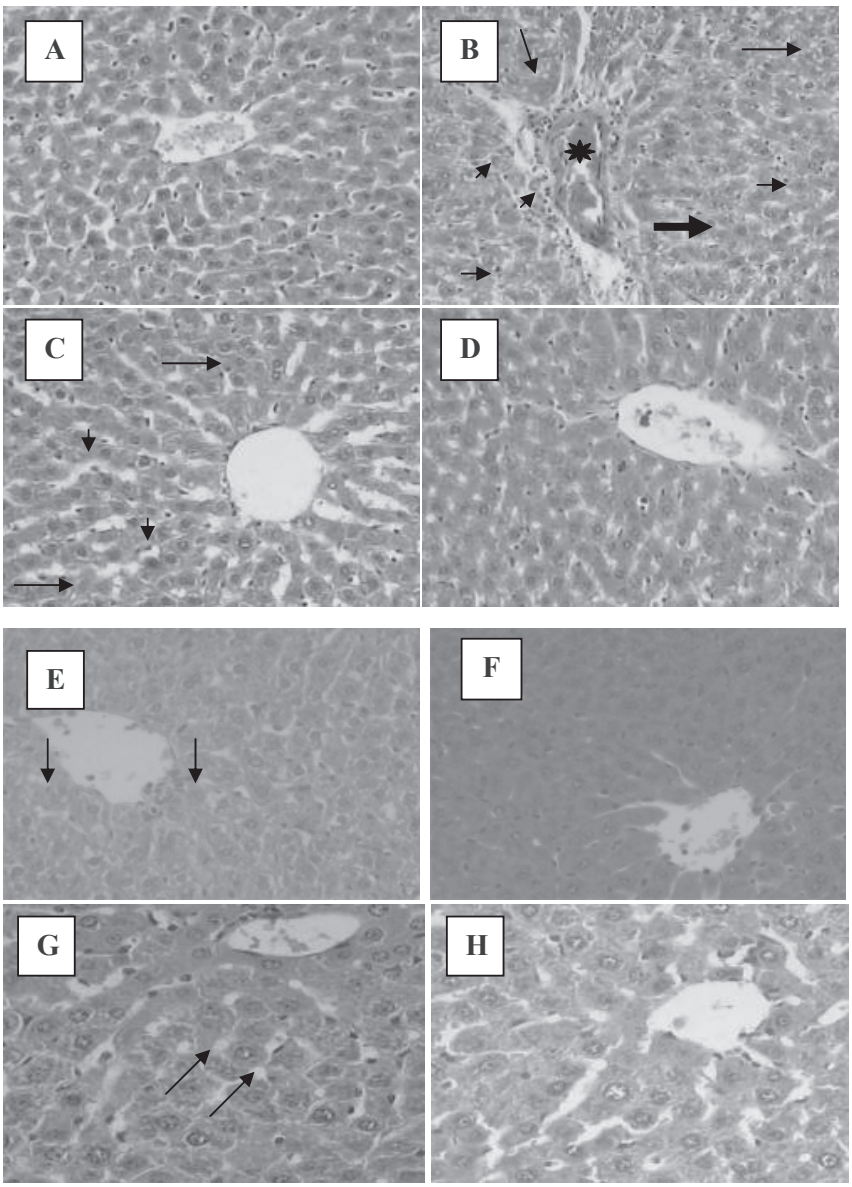

Fig. 2. (A) Liver of control mice showing normal histology. (B) Acetaminophen-induced hepatotoxicity showing extensive areas of necrosis (arrow), hydropic degeneration (thick arrow). Congested portal tract (asterisk) associated with inflammatory cells (arrow heads) in portal and periportal, congested sinusoids (two arrow heads) and hepatocytes with pyknotic nuclei (small arrow) are also seen. (C) Pre-treatment with 25 mg/kg BW of *L. camara* extract showing partial protection of hepatocytes. Notice: necrosis of single hepatocytes (arrows) and activated Kuppfer cell (arrow heads). (D) Pre-treatment with 75 mg/kg BW of *L. camara* extract showing hepatocytes that appear more or less normal. (E) Post-treatment with 25 mg/kg BW of *L. camara* extract showing foci of necrotic hepatocytes (arrows). (F) Post-treatment with 75 mg/kg BW of *L. camara* extract showing hepatocytes that appear more or less normal. (G) Pre-treatment with silymarin (50 mg/kg BW) showing few vacuoles (arrows) and activated Kuppfer cells (arrows). (H) Post-treatment with silymarin (50 mg/kg BW) showing hepatocytes that appear more or less normal. Hematoxylin and eosin stain, (A - F) x 150, (G, H) x 300.

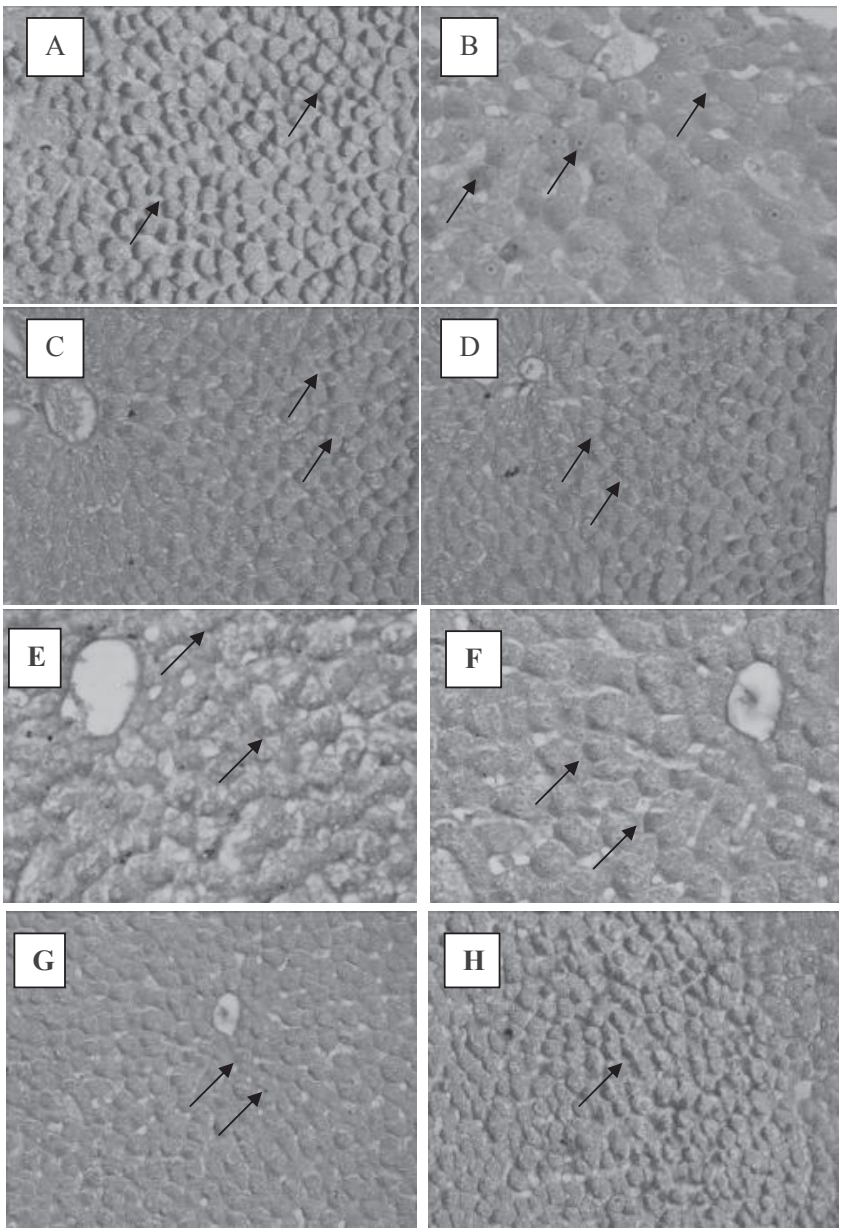

Fig. 3. (A) Liver of control mice showing normal distribution of glycogen (arrows). (B) Acetaminophen-induced moderate depletion of glycogen in the hepatocytes (arrows). (C) Pre-treatment with 25 mg/kg BW of *L. camara* extract showing moderate increase in hepatocyte glycogen (arrows) as compared to the acetaminophen group. (D) Pre-treatment with 75 mg/kg BW of *L. camara* extract showing hepatocyte glycogen (arrows) that appears similar to the control group. (E) Post-treatment with 25 mg/kg BW of *L. camara* extract showing a mild increase in hepatocyte glycogen (arrows) as compared to the acetaminophen group. (F) Post-treatment with 75 mg/kg BW of *L. camara* extract showing hepatocyte glycogen (arrows) that appears more or less like in the control group. (G) Pre-treatment with silymarin (50 mg/kg BW) showing a mild increase in hepatocyte glycogen (arrows) as compared to the acetaminophen group. (H) Post-treatment with silymarin (50 mg/kg BW) showing hepatocyte glycogen (arrow) that appears like in the normal group. Periodic acid-Schiff stain, (A, C, D, G, H) x 150, (B, E, F) x 300.

were performed on a Jeol ECA-500 NMR spectrometer (Tokyo, Japan) using DMSO-d_6 as solvent. All chemical shifts (δ) are given in ppm with reference to tetramethylsilane (TMS) as an internal standard; the coupling constants (J) are given in Hz. Paper chromatography (PC, Whatman No. 1 & 3 MM, Kent, England) was done using the following solvent systems: H_2O; HOAc/H_2O (15:85, v/v); BAW (*n*-BuOH/HOAc/H_2O, 4:1:5, v/v/v, upper layer). Mixture solvents (BAW) were used for sugar analysis.

Plant material

Yellow flowers of *Lantana camara* L. were collected from Al-Orman Garden, Cairo, Egypt, in March 2009, and authenticated by Dr. Mohammed El-Gibaly, consultant of plant taxonomy, Faculty of Pharmacy, Cairo University, Cairo, Egypt. A voucher specimen (No. 801) was deposited at the National Research Centre Herbarium, Cairo, Egypt.

Extraction and purification

The air-dried powdered flowers of *L. camara* (900 g) were exhaustively extracted with 70% aqueous methanol (3 × 3 l) at room temperature, and the solvent was evaporated under reduced pressure. The resulting residue was suspended in water and successively partitioned in *n*-hexane (3 × 100 ml) and dichloromethane (3 × 300 ml). The aqueous layer was evaporated to dryness under reduced pressure. The residue (80 g) was applied to a polyamide column (100 cm × 5 cm) eluted with water followed by increasing percentages of methanol (up to 100%). A total of 120 fractions were collected (100 ml each). These were combined in seven fractions according to PC analysis on Whatman filter paper sheets (1 MM). Fraction I was chromatographed on a cellulose column using MeOH/H_2O (8:2, v/v) to yield compounds **1** (22 mg) and **2** (14 mg), while fraction II was purified on Sephadex LH-20 using MeOH/H_2O (1:1, v/v) to give compound **3** (28 mg). Fraction III was further separated using preparative PC (Whatman No. 3 MM) with HOAc/H_2O (15:85, v/v) followed by Sephadex LH-20 chromatography using MeOH as eluent to give compounds **4** (12 mg) and **5** (7 mg). Fraction IV was purified on a Sephadex LH-20 column using MeOH/H_2O (1:1, v/v) to give compound **6** (15 mg). Compounds **7** (15 mg) and **8** (12 mg) were separated from fraction V on a cellulose column using MeOH/H_2O (1:1, v/v). Fractions VI and VII were subjected directly to a Sephadex LH-20 column using MeOH/H_2O (1:1, v/v) to yield compounds **9** (8 mg) and **10** (12 mg), respectively.

Luteolin 7-O-β-galacturonyl-(2→1)-O-β-galacturonide (**1**): Normal acid hydrolysis gave galacturonic acid (CoPC) and luteolin. – UV: λ_{max} (MeOH) = 257, 268, 351; (+NaOMe) 265, 402; (+NaOAc) 257, 266, 351; (+NaOAc/H_3BO_3) 259, 372; (+$AlCl_3$) 274, 297, 425; (+$AlCl_3$/HCl) 272, 296, 357, 389 nm. – (+)-ESI MS: m/z = 639 $[M+H]^+$. – ^{1}H NMR and ^{13}C NMR (DMSO-d_6): see Table I.

Apigenin 7-O-β-galacturonyl-(2→1)-O-β-galacturonide (**2**): Normal acid hydrolysis gave galacturonic acid (CoPC) and apigenin. – UV: λ_{max} (MeOH) = 268, 331; (+NaOMe) 267, 388; (+NaOAc) 267, 338; (+NaOAc/H_3BO_3) 267, 335; (+$AlCl_3$) 275, 297, 344, 379; (+$AlCl_3$/HCl) 275, 297, 340, 381 nm. – (+)-ESI MS: m/z = 623. – ^{1}H NMR and ^{13}C NMR (DMSO-d_6): see Table I.

Acid hydrolysis

Acid hydrolysis was performed using 1% aqueous HCl at 100 °C for 75 min. For aglycone detection, the final mixture was extracted with EtOAc, then the aqueous layer was neutralized for determination of the released sugar moieties using silica gel plates with *n*-PrOH/EtOAc/H_2O (7:2:1, v/v/v); aniline phthalate was employed as a spray for colour detection of the sugars.

Sugar identification by HPLC

A sample of 50 μl of the aqueous layer of the hydrolysis solution was chromatographed using a 3-mm Luna C18 column (Shodex) eluted with water (isocratic mobile phase) for 30 min at a flow rate of 1.0 ml/min. There was an 1-min delay before the next injection. A refractive index (RId-10A) detector was used.

Free radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid was purchased from Merck (Darmstadt, Germany). The stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and then stored at –20 °C until needed.

The free radical scavenging activity was measured using the method of Shimada *et al.* (1992). All extracts and isolated compounds were

initially screened at 100 μg/ml. The most potent ones (more than 90% radical scavenging) were assayed at the concentrations 25, 50, and 75 μg/ml to calculate IC_{50} values. Briefly, an 0.1 mM solution of DPPH in methanol was prepared. One ml of this solution was added to 3 ml of extract or solution of isolated compound at 25, 50, and 75 μg/ml. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a microplate reader.

Percent scavenging of DPPH free radicals was measured using the following equation: DPPH scavenging effect (%) = $100 - [(A_0 - A_1)/A_0) \cdot 100]$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample (Oktay *et al*., 2003).

Experimental animals

Male mice (22–25 g) bred in the Lab Animal House, National Research Centre, Cairo, Egypt, were used. The mice were kept in standard environmental conditions (23–25 °C and 12 h light/12 h dark cycle) and were fed on a standard pellet diet and water *ad libitum*. Eight groups of mice, each consisting of six mice, were used. Group I served as control, group II received a single administration of acetaminophen was given (3 g/kg BW), groups III and IV received the extracts of *L. camara* (25 and 75 mg/kg BW per day) for 7 d before a single dose of acetaminophen was given. Groups V and VI received the extracts of *L. camara* (25 and 75 mg/kg BW per day) for 7 d after a single dose of acetaminophen. Groups VII and VIII received silymarin (50 mg/kg BW per day) for 7 d before and after administration of a single dose of acetaminophen, respectively. The experimental protocols of this study were performed in accordance with the guidelines for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, National Research Centre, Cairo, Egypt.

Assessment of liver functions

Twenty-four h after toxin administration, the mice of each group were anaesthetized, and blood was collected directly from the heart. The blood samples were allowed to clot for 20–30 min. Serum was separated by centrifugation at 37 °C and used for estimation of various biochemical parameters. Values are presented as means ± S.E., and the percent protection in individual biochemical parameters from their elevated values caused by acetaminophen was calculated.

Assay of serum transaminases

The activities of serum glutamate oxaloacetate transaminase (SGOT) (EC 2.6.1.1) and serum glutamate pyruvate transaminase (SGPT) (EC 2.6.1.2) were estimated by the method of Reitman and Frankel (1957). The enzyme activity was expressed as IU/l.

Assay of alkaline phosphatase

The activity of serum alkaline phosphatase (ALP) (EC 3.1.3.1) was estimated by the method of Kind and King (1954). The enzyme activity was expressed as IU/l.

Histopathological and histochemical studies

After the animals had been sacrificed, their livers were carefully dissected. The livers were rinsed in normal saline and sections were taken from them. The sections were fixed in 10% formalin, dehydrated with an ascending gradient of ethanol solution (30, 50. 70, 80, 90, and 100%) and embedded in paraffin. Five μm thick sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) and observed under a photomicroscope for histopathological and histochemical investigation. Other specimens were fixed in Carnoy's fixative and embedded in paraffin. The PAS method was used for glycogen staining (Hotchkiss, 1948).

Statistical analyses

Values of the biochemical parameters are reported as means ± S.E. Total variation, present in a set of data, was estimated by one way analysis of variance (ANOVA); Student's *t*-test was used for determining significance (Woolson, 1987).

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