HEPATOPROTECTIVE EFFECT OF BAY LEAVES CRUDE EXTRACT ON PRIMARY CULTURED RAT HEPATOCYTES

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Abstract:
The crude extract of bay leaves (*Laurus nobilis*) grown in Egypt, was tested for its hepatoprotective activity on primary cultured rat hepatocytes against paracetamol toxicity. The Neutral Red Assay was used to assess the hepatotoxicity of the extract and was applied with a broad range of concentrations (125-1000 µg/ml) on monolayer of rat hepatocytes. It revealed that the extract exerts no toxic effect on the monolayer hepatocytes. For the evaluation of the hepatoprotective effect; different concentrations were prepared, starting from 12.5 µg/mL and increasing concentration in ascending order by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out, in addition to controls which were: cell control (cells only), negative control (cells + paracetamol) and positive control (reference) (cells + sylimarin + paracetamol). 50% mortality of the hepatocytes (IC_{50}) was determined using Neutral Red Assay. *L. nobilis* extract showed hepatoprotective activity against paracetamol toxic effect at concentration of 40 µg/ ml. The constitutive flavonoids of *L. nobilis* leaves were extensively studied and led to the separation and identification of 14 compounds, 10 of which have not been previously identified in *L. nobilis* and were identified using chemical, conventional and advanced spectral techniques.

Key Words: *Laurus nobilis;* Lauraceae; flavonoids; hepatoprotective

Introduction:

Laurus nobilis (Lauraceae), known in English as bay laurel, sweet bay, bay tree, true laurel, or Grecian laurel (Brown, 1956) is an aromatic evergreen tree or large shrub with green, glossy leaves, native to the Mediterranean region. It has been analyzed for alkaloids (Pech and Bruneton ,1982), essential oils (Sellami I.H. *et al*. 2011, Zola *et al*., 1977), acylated kaempferol glycosides (Fiorin C. *et al*., 1998), sesquiterpene lactones (Dall'Acqua *et al*. 2006), megastigmane glycosides (Marino *et al*., 2004), (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (+)-epigallocatechin and procyanidins (B2, B4, B5 and B7) (Sakar M. K. *et al*, 1985). The plant is one of the most widely used culinary spices in all Western countries. Its leaves are traditionally used orally to treat the symptoms of gastrointestinal problems, such as epigastric bloating, impaired digestion, eructations and flatulence (Brunetton, 1999). The aqueous extract is used in Turkish folk medicine as an anti-hemorrhoidal, anti-rheumatic, diuretic, as an antidote in snake bites and for the treatment of stomach-ache (Sezik E., 1999, Baytop T., 1984).

A survey of the literature showed that no experimental data are available regarding its hepatoprotective potential. It was therefore, interesting to investigate, in the present study the possible hepatoprotective effect of *L. nobilis* leaves and to separate and identify some of their constitutive flavonoids.

Experimental:

***Plant Material*:**

The leaves of *L. nobilis* were collected from a private farm in El- Mansouria area, Giza, Egypt, in September 2010 and authenticated by Dr. M. El Gebali, National Research Centre (NRC), Cairo , Egypt.

***Instruments and Materials*:**

^{1}H NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts relative to DMSO-d_6 and converted to TMS scale by adding 39.5. spectral width = 8 kHz for ^{1}H and 30 kHz for ^{13}C, 64 K data points and a flip angle of 45^o. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Paper chromatographic analysis was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6 % HOAc; (3) BAW (n-BuOH : HOAc : H_2O, 4:1:5, upper layer). Solvent 3 was used for preparative paper chromatography (Prep. PC) using Whatman No. 3 paper.

***In vitro Bioassay on Primary Culture of Rat Hepatocytes Monolayer*:**

***Isolation and Culture of Rat Hepatocytes Monolayer*:**

Primary culture of rat hepatocytes was prepared according to (Seglen, 1976) method, modified by (Kiso, *et al.* 1983) using Wistar male rats (250-300 g) obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). At time of surgery, the rat was anesthesized by intra-peritoneal injection of 3.4mL/kg of sodium thiopental solution (0.1g/mL). A midline incision was made, the liver was exposed and the portal vein was cannulated with a needle fitted with a teflon catheter. After the teflon catheter was tied in place and the needle removed, the inferior *vena cava* was cut below the renal vein to allow blood drainage. Perfusion of the liver was started with Ca^{2+} free buffer (buffer 1), which contained HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (10 mmole/L), NaCl (137 mmole/L), KCl (2.68 mmole/L), Na_2HPO_4 (0.7 mmole/L), D (+) glucose (10 mmole/L) and EGTA (ethylene glycol tetraacetic acid) (0.5 mmole/L), and adjusted to pH 7.45 at 37ºC. The flow-rate was 11.6 mL/min. The thoracic portion of the superior *vena cava* was cannulated, and the inferior *vena cava* was tied-off above the renal vein. After perfusing the liver for 15min., recirculation of Ca^{2+}-containing buffer (buffer 2), containing additionally to buffer 1, $CaCl_2$ (5mmole/L) and Collagenase type IV (10g/L) (pH 7.45 at 37ºC) was started, adjusted at a flow-rate of 7.5 mL/min, for 10 min. After perfusion with buffer 2, the liver was dissected out of the body, placed in a beaker containing buffer 2 and transferred under sterile conditions to laminar flow and gently dispersed with two forceps into small pieces (2-3 mm). The cell suspension was then filtered through the cotton gauze into centrifuge tubes. Finally, the preparation was centrifuged at 600 rpm for 5 min. The supernatant was aspirated off and the loosely packed pellet of cells was gently re-suspended in Phosphate buffer (PB) (0.1 mole/L), which contained NaH_2PO_4 (0.2 mole/L) and Na_2HPO_4 (0.2 mole/L), adjusted at pH 7.6 at 37ºC, and then diluted two-folds with distilled water. This washing procedure was repeated twice. Viability of the cells to exclude the dye, trypan blue, was determined by incubating cell suspension (0.1 mL) with trypan blue (0.9 mL) and then counting the number of the cells that excluded the dye and the number of cells whose nuclei were stained blue, using haemocytometer under light microscope (Nikon). Complete culture medium was added over the total counted cells to reach a concentration of 1 x 10^6 cells/mL.

The culture medium was composed of RPMI-1640 medium, supplemented with 10% inactivated (56ºC for 30 min) fetal calf serum (FCS) (0.05/mL), penicillin-streptomycin (0.01/mL), insulin (0.7 (g/mL) and dexamethasone (4g/mL). Inocula of 1 x 10^6 cells/mL were seeded into plastic 96-well plates and preincubated in CO_2-Incubator at 37ºC, under 5% CO_2 in air for 22-24 hrs. All buffers were freshly prepared and sterilized at 121ºC for 30 min. before use.

***IC_{50} Determination of the extract*:**

After pre - incubation of primary culture of rat hepatocytes for 22-24 hrs, the monolayer was checked under inverted microscope (Olympus) for attachment. Then, the monolayer was washed twice with (Phosphate Buffer Saline (PBS), which contained KCl (2.68 mmole/L), Na_2HPO_4 (8.45 mmole/L), KH_2PO_4 (1.5 mmole/L) and NaCl (137 mmole/L).

In order to determine IC_{50}, different concentrations of the extract were prepared. The range of concentrations used started from 125 µg/mL followed by increasing concentrations in ascending order up to the concentration 1000 µg/mL that induced death of half the number of cells. *L. nobilis* extract was dissolved in dimethylsulfoxide (DMSO) (1% maximum concentration). For each concentration, three replicates were carried out. The plate was incubated for 2 hrs, in CO_2 incubator.

After cell incubation with the extract, cell viability was determined using neutral red assay (NR). 200 µL culture medium containing all except (PS), was added to each well together with 50 µL of a solution of 2 mg NR/mL PBS. After incubation for 3 hrs, supernatant was removed as previously described and the neutral red dye obtained was dissolved in 200 µL 50% ethanol with 1% acetic acid. The plate was then shaken for 5 min. on a microtitre plate shaker and the plate on Microelisa reader was read at 540 and 630 nm dual wavelength using automatic kinetic microplate reader (Labsystems Multiskan RC reader).

Evaluation of the effect of the extract on cultured hepatocytes was obtained by calculating the absorption of the cell viability with respect to control cells. Each experiment was carried out in triplicate to confirm validity of results. A graph was plotted with x-axis showing different concentrations of extract used, y-axis showing absorbance percentage of viable cells. IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with 50% absorbance.

Extraction, isolation and purification:

The aqueous methanolic extract of *L. nobilis* leaves (48 g) showed by preliminary two-dimensional paper chromatographic (2D-PC) screening to contain a complicated flavoniod mixture from which fourteen compounds (**1-14**) were isolated and purified through fractionation on polyamide 6s (400 g) column (150 × 5 cm) and elution with H_2O followed by H_2O-MeOH mixtures of decreasing polarities to yield eight column fractions (I-VIII). The received fractions, were individually subjected to 2D-PC and other isolatory chromatographic techniques, thus yielding ten flavonoids **(1, 4, 5-12)** isolated from this plant for the first time.

Isolation and purification of flavonoids from *L. nobilis* leaves.

Compound **1** (87 mg) was purely isolated from fraction II (eluted with 10% aq. MeOH) by repeated column fractionation of 1.8 g material over Sephadex LH-20 using H_2O for elution. Compound **2** (108.8 mg) was crystallized from an aqueous solution of 2.1 g of fraction III (eluted with 20% aq. MeOH). Compound **3** (99 mg) was purely isolated from 1.1 g of fraction IV (eluted with 30% aq. MeOH) by preparative PC, using BAW as solvent. Compounds **4** (76 mg), **5** (115 mg) and **6** (78.5 mg) were individually separated pure from a Sephadex LH-20 column of fraction V (2.2 g, eluted with 50% aq. MeOH), using n-butanol water saturated for elution. Compounds **7** (54 mg), **8** (71 mg) and **9** (57 mg) were obtained pure from 1.93 g of fraction VI (eluted with 60% aq. MeOH) by applying Sephadex LH-20 column fractionation, using n-butanol water saturated for elution. Compound **10** (31 mg) and **11** (66 mg) were obtained from 3.1 g of fraction VII (eluted with 80% aq. MeOH). Compounds **12** (74 mg) and **13** (35 mg) and **14** (20mg) were isolated from 6.2 g of fraction VIII (eluted with 90% aq. MeOH) by repeated prep. PC using 6% AcOH and BAW as solvent.

Spectral data of compounds (1, 4, 5-12) isolated for the first time from *L.nobilis* leaves.

Quercetin 3-*O*- α-L-rhamnopyranoside (1) was obtained as pale yellow amorphous powder of R_f values (x 100):22 (H_2O), 48 (AcOH-6), 68(BAW). It exhibited a *Mr* of 448 in negative ESI-MS analysis ($[M-H]^-$ at *m/z* = 447.1). UV spectral data λmax (MeOH): 259 nm, 297 nm sh., 348 nm, + NaOMe: 270 nm, 355nm, 402 nm, + NaOAC: 276 nm, 372 nm, + NaOAC + H_3BO_3: 272 nm, 383 nm, +Al Cl_3: 268 nm, 352 nm, 408 nm. ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 6.17 (d, *J*=2.5 Hz, H-6), 6.36 (d, *J*=2.5 Hz, H-8), 7.25 (d, *J*=2.5, H-2'), 6.82 (d, *J*=8 Hz, H-5'), 7.25 (dd, *J*=2.5 and 8 Hz, H-6'), rhamnose moiety: 5.20 ($\Delta\nu_{1/2}$ = 4 Hz, H-1"), 3.1 – 3.9 (m, overlapped with water proton resonances, H-2"- H-5"), 0.77 (d, *J*= 6, H-6''). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 156.9 (C-2), 134.6 (C-3), 178.2 (C-4), 161.7(C-5), 99.19 (C-6), 164.7 (C-7), 94.15(C-8), 157.8 (C-9), 104.5 (C-10),121.2 (C-1'),115.9 (C-2'), 145.7 (C-3'), 148.9(C-4'), 116.1(C-5'), 121.6 (C-6'), rhamnose moiety: 102.2 (C-1"), 70.8 (C-2"), 71.1 (C-3"), 71.6 (C-4"), 70.5 (C-5"), 18.01(C-6'').

Kaempferol 3-*O*- β- glucopyranoside (4): was obtained as pale yellow amorphous powder of R_f values (x 100): 24 (H_2O), 47 (AcOH-6), 76 (BAW). It exhibited a *Mr* of 432 in negative ESI-MS analysis $[M-H]^-$ at *m/z* = 431. UV spectral data λmax (MeOH): 266 nm, 345 nm, + NaOMe: 271 nm, 376nm, + NaOAC: 270 nm, 346 nm, + NaOAC + H_3BO_3: 270 nm, 346 nm sh., 405, +Al Cl_3: 268 nm, 340 nm sh., 385 nm. ^{1}H- NMR spectral rata (DMSO-d_6) δ (ppm), kaempferol moiety: 6.186 (d, *J*=2.5 Hz,H-6), 6.38 (d, *J*=2.5 Hz,H-8), 7.7 (d, *J*=2.5, H-2', H-6'), 6.89 (d, *J*=8 Hz, H- 3',H-5'), glucose moiety: 5.26 (d, *J*=8 Hz, H-1''), 3.4-4.0 (m, H-2''- H-6''). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), kaempferol moiety: 158.6 (C-2), 133.6 (C-3), 179.9 (C-4), 162.5 (C-5), 100.9 (C-6), 167.0 (C-7), 95.2 (C-8), 159.3 (C-9), 106.1 (C-10), 123.1 (C-1'), 132.6 (C-2', C-6'), 116.3 (C-3', C-5'), 162.1 (C-4'), glucose moiety: 105.1 (C-1''), 73.1 (C-2''), 75.1 (C-3''), 70.3 (C-4''), 77.2 (C-5''), 61.9 (C-6'').

Quercetin 3'-O- β- glucopyranoside (5) was obtained as yellowish white amorphous powder of R_f values (x 100): 23 (H_2O), 42 (AcOH-6), 53 (BAW). It showed a M*r* of 464 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 463. UV Spectral Data λ_{max} (MeOH): 255 nm, 266 nm sh., 370 nm + NaOMe: 271 nm, 330 nm, 430 nm, + NaOAC: 260 nm sh., 272 nm, 320 nm, 340, + NaOAC + H_3BO_3 : 255 nm, 268 nm sh., 305 nm sh., 375 nm, +Al Cl_3 : 265 nm, 305 nm sh., 360 nm , 430 nm. ^{1}H- NMR Spectral Data (DMSO-d_6) δ (ppm), quercetin moiety: 6.16 (d, *J*=2.5 Hz, H-6), 6.6.4 (d, *J*=2.5 Hz, H-8), 6.86 (d, *J* = 8.0 Hz, H-5'), 7.45 (dd, *J*=2.5 and 7.5 Hz, H-6'), 7.85 (s, H- 2'), glucose moiety: 5.05 (d, *J*=8 Hz, H-1''), 3.4-4.0 (m, H-2''- H-6''). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety:147.4 (C-2), 137.47 (C-3), 177.36 (C-4), 162.53 (C-5), 99.3 (C-6), 165.65 (C-7), 94.42 (C-8), 158.17 (C-9), 104.55 (C-10), 124.92 (C-1'), 115.86 (C-2'), 147.67 (C-3'), 146.52 (C-4'), 116.97 (C-5'), 121.42 (C-6'), glucose moiety: 105.1 (C-1''), 73.1 (C-2''), 75.1 (C-3''), 70.3 (C-4''), 77.2 (C-5''), 61.9 (C-6'').

Quercetin 3-*O*-*β*-galactoside (6) was obtained as yellowish white amorphous powder of R_f values (x 100): 20 (H_2O), 37 (AcOH-6), 56 (BAW). It showed a M*r* of 464 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 463. UV spectral data λ_{max} (MeOH): 257 nm, 266 nm sh., 359 nm, + NaOMe: 266 nm, 227 nm sh., 408 nm, + NaOAC: 274 nm, 379 nm, + NaOAC + H_3BO_3: 268 nm, 272 nm sh., 384 nm, +Al Cl_3: 265 nm, 272 nm sh., 384 nm. ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 6.19 (d, *J*=2.5 Hz, H-6), 6.4 (d, *J*=2.5 Hz, H-8), 7.54 (d, *J*=2.5 Hz, H-2'), 6.82 (d, *J*=8 Hz, H-5'), 7.65 (dd, *J*=2 and 8 Hz, H-6'), galactose moiety: 5.36 (d, *J*=8 Hz, H-1"), 3.2 – 3.8 (m, H2"-6"). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 156.24 (C-2), 133.33 (C-3), 177.32 (C-4), 160.57 (C-5), 98.61(C-6), 164.79 (C-7), 93.57 (C-8), 156.26 (C-9), 103.75 (C-10), 120.91(C-1'), 115.164 (C-2'), 144.89 (C-3'), 148.31 (C-4'), 115. 51 (C-5'), 121. 81(C-6'), galactose moiety: 101.79 (C-1"), 71.074 (C-2"), 72.97 (C-3"), 67.81 (C-4"), 75.54 (C-5"), 60 (C-6").

Isorhamnetin 3-*O*- β- glucopyranoside (7) was obtained as yellowish white amorphous powder of R_f values (x 100): 11 (H_2O), 12 (AcOH-6), 52 (BAW). It showed a M*r* of 478 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 477.3. UV spectral data λ_{max} (MeOH): 256 nm, 300 nm sh., 354 nm, + NaOMe : 275 nm, 325 nm sh., 408 nm, + NaOAC: 275 nm, 323 nm, 371 nm, + NaOAC + H_3BO_3 : 276 nm, 295 nm sh., 355 nm +Al Cl_3 : 269 nm, 365 nm, 403 nm. ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 6.22 (d, *J*=1.6 Hz, H-6), 6.43 (d, *J*=1.6 Hz, H-8), 3.84(s, OCH_3), 7.95 (d, *J*=1.6 Hz, H-2'), 6.93 (d, *J*=8.4 Hz, H-5'), 7.56 (dd, *J*=1.6 and 8.4 Hz, H-6'), glucose moiety: 5.57 (d, *J*=8 Hz, H- 1''), 3.0-3.8 (m, H-2''- H-6''). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 156.3 (C-2), 133.2 (C-3), 177.3 (C-4), 161.1 (C-5), 98.7 (C-6), 164.3 (C-7), 93.6 (C-8), 156.2 (C-9), 103.9 (C-10), 55.6 (OCH_3), 120.9 (C-1'), 113.4 (C-2'), 148.4 (C-3'), 149.3 (C-4'), 115.1 (C-5'), 122.0 (C-6'), glucose: 100.8 (C-1"), 74.2 (C-2"), 76.3 (C-3"), 70.4 (C-4"), 77.3 (C-5"), 60.5 (C-6").

Isorhamnetin 3-*O*- β- galactopyranoside (8) was obtained as yellowish white amorphous powder of R_f values (x 100): 11 (H_2O), 12 (AcOH-6), 52 (BAW). It showed a M*r* of 478 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 477.3. UV spectral data λ_{max} (MeOH): 256 nm, 300 nm sh., 354 nm, + NaOMe : 275 nm, 325 nm sh., 408 nm, + NaOAC: 275 nm, 323 nm, 371 nm, +

NaOAC + H_3BO_3 : 276 nm, 295 nm sh., 355 nm, +Al Cl_3 : 269 nm, 365 nm, 403 nm. ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 6.22 (d, *J*=1.6 Hz, H-6), 6.43 (d, *J*=1.6 Hz, H-8), 3.84(s, OCH_3), 7.95 (d, *J*=1.6 Hz, H-2'), 6.93 (d, *J*=8.4 Hz, H-5'), 7.56 (dd, *J*=1.6 and 8.4 Hz, H-6'), galactose moiety: 5.36 (d, *J*=8 Hz, H- 1''), 3.2 - 3.8 (m, H-2''- H-6''). ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 156.3 (C-2), 133.2 (C-3), 177.3 (C-4), 161.1 (C-5), 98.7 (C-6), 164.3 (C-7), 93.6 (C-8), 156.2 (C-9), 103.9 (C-10), 55.6 (OCH_3), 120.9 (C-1'), 113.4 (C-2'), 148.4 (C-3'), 149.3 (C-4'), 115.1 (C-5'), 122.0 (C-6'), glalactose: 101.79 (C-1"), 71.07 (C-2"), 72.97 (C-3"), 67.81 (C-4"), 75.54 (C-5"), 60.5(C-6").

Quercetin 3-*O*- rutinoside (9) was obtained as yellowish white amorphous powder of R_f values (x 100): 48 (H_2O), 44 (AcOH-6), 32 (BAW). It showed a M*r* of 610 in negative ESI-MS, corresponding to a molecular ion [M-H]$^-$ at *m/z* = 609.1. UV spectral data λ_{max} (MeOH): 256 nm, 265 nm sh., 358 nm+ NaOMe : 268 nm, 327 nm sh., 403 nm, + NaOAC: 273 nm, 323 nm, 387 nm, + NaOAC + H_3BO_3 : 262 nm, 377 nm, +Al Cl_3 : 273 nm, 430 nm. ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 6.0 (d, *J*=2.5 Hz, H-6), 6.2 (d, *J*=2.5 Hz, H-8), 7.4 (d, *J*=2.5 Hz, H-2'), 6.7 (d, *J*=8 Hz, H-5'), 7.5(dd, *J*=2 and 8 Hz, H-6'), glucose moiety: 5.23 (1H, d, *J* = 7.5 Hz, H-1"), 3-3.8 (m, H-2"- H- 6"), rhamnose moiety: 4.37 (s, H-1'"), 3-3.8 (m, H-2'"- H- 5'"), 1.10 (d, *J* = 6.0 Hz, H-6'") . ^{13}C-NMR spectral data (DMSO-d_6) δ (ppm), quercetin moiety: 156.9 (C-2), 133. 8 (C-3), 177.8 (C-4), 161.7 (C-5), 98.7(C-6), 164.6 (C-7), 93.9 (C-8), 157.1 (C-9), 104.4 (C-10), 121.6 (C-1'), 115.7 (C-2'), 145.3 (C-3'), 149.0 (C-4'), 116.7 (C-5'), 122.3(C-6'), glucose moiety: 101.2 (C-1''), 74.59 (C-2''), 76.9 (C-3''), 70.5 (C-4''), 76.4 (C-5''), 67.5 (C-6''), rhamnose moiety: 101.7 (C-1'"), 70.8 (C-2"'), 71.1 (C-3,'"), 72.36 (C-4'"), 68.7 (C-5'"), 18.4(C-6''').

Kaempferol 3-O- rutinoside (10) was obtained as yellowish white amorphous powder of R_f values (x 100): 49 (H_2O), 47 (AcOH-6), 35 (BAW). It showed a M*r* of 594 in negative ESI-MS, corresponding to a molecular ion [M-H]$^-$ at *m/z* = 593.1. UV Spectral Data λ_{max} (MeOH): 252 nm, 352 nm sh., + NaOMe : 273 nm, 324 nm sh., 395 nm, + NaOAC: 273 nm, 310 nm, 385 nm, + NaOAC + H_3BO_3 : 266 nm, 355 nm, +$AlCl_3$: 272 nm, 392 nm. ^{1}H- NMR Spectral Data (DMSO-d_6) δ (ppm), kaempferol moiety: 6.18 (d, *J*=2.5 Hz, H-6), 6.44 (d, *J*=2.5 Hz, H-8), 7.92 (d, *J*=2.5 Hz, H-2', H-6'), 6.82 (d, *J*=8 Hz, H- 3',H-5'), glucose moiety: 5.23 (d, *J* = 7.5 Hz, H-1"), 3-3.8 (m, H-2"- H- 6"), rhamnose moiety: 1.10 (1H, d, *J* = 6.0 Hz, H-6'"), 4.37 (s, H-1'"), 3-3.8 (m, H-2'"- H- 5'"). ^{13}C-NMR Spectral Data (DMSO-d_6) δ (ppm), kaempferol moiety: 157.4 (C-2), 133.7 (C-3), 178.2 (C-4), 161.5 (C-5), 99.4 (C-6), 164.9 (C-7), 94.3 (C-8), 157.8 (C-9), 104.4 (C-10), 121.3 (C-1'), 131.7 (C-2', C-6'), 161.8 (C-4'), 115.8 (C-3', C-5'), glucose moiety: 100.9 (C-1''), 74.59 (C-2''), 76.9 (C-3''), 70.5 (C-4''), 76.4 (C-5''), 67.5 (C-6''), rhamnose moiety: 102.2 (C-1'"), 70.8 (C-2'''), 71.1 (C-3'"), 71.6 (C-4'"), 70.5 (C-5'"), 18.01(C-6''').

Isorhamnetin-3-*O*-rutinoside (11) was obtained as yellowish white amorphous powder of R_f values (x 100): 22 (H_2O), 39 (AcOH-6), 48 (BAW). It showed a M*r* of 624 in negative ESI-MS, corresponding to a molecular ion [M-H]$^-$ at *m/z* = 623. UV Spectral Data λ_{max} (MeOH): 252 nm, 352 nm sh., + NaOMe : 268 nm, 326 nm sh., 405 nm, + NaOAC: 269 nm, 322 nm, 360 nm, + NaOAC + H_3BO_3 : 252 nm, 353 nm sh., +Al Cl_3 : 264 nm, 298 nm sh., 355 nm. ^{1}H- NMR Spectral Data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 3.97 (s, OCH3), 6.15 (d, *J*=2.5 Hz,H-6), 6.35 (d, *J*=2.5 Hz,H-8), 6.93 (d, *J* = 8.0 Hz, H-5'), 7.5 (d, *J* = 8.0 Hz, H-6'), 7.85 (s, H- 2'), glucose moiety: 5.4 (d, *J* = 7.5 Hz, H-1"), 3-3.8 (m, H-2"- H- 6"), rhamnose moiety: 4.53 (s, H-1'"), 3-3.8 (m, H-2'"- H- 5'"), 1.10 (d, *J* = 6.0 Hz, H-6'"). ^{13}C-NMR Spectral Data (DMSO-d_6) δ (ppm), isorhamnetin moiety: 157.4 (C-2), 134.3 (C-3), 161.9 (C-5), 98.9 (C-6), 165.1 (C-7), 93.8 (C-8), 157.7 (C-9), 104.5 (C-10), 122.7 (C-1'), 113.4 (C-2'), 147.2 (C-3'), 55.6 (C-3'-OCH3), 149.6 (C-4'), 114.9 (C-5'), 121.8 (C- 6'), glucose moiety: 103.0 (C-1"), 74.7 (C-2"), 77.0 (C-3"), 70.4 (C-4"),76.2 (C-5"), 67.3 (C-6"), rhamnose moiety: 101.3 (C-1'"), 70.8 (C-2'"), 70.9 (C-3'"), 72.7 (C-4'"), 68.6 (C-5'"), 16.7 (C-6'").

Isorhamnetin (12) was obtained as yellowish white amorphous powder of R_f values (x 100): 0 (H_2O), 2 (AcOH-6), 73 (BAW). It showed a M*r* of 316 in negative ESI-MS, corresponding to a molecular ion [M-H]$^-$ at *m/z* = 315. UV Spectral Data λ_{max} (MeOH): 255 nm, 266 nm sh., 370 nm + NaOMe : 271 nm, 330 nm, 430 nm, + NaOAC: 260 nm sh., 272 nm, 320 nm, 340, + NaOAC + H_3BO_3 : 255 nm,

268 nm sh., 305 nm sh., 375 nm, $+Al_3\ Cl_3$: 265 nm, 305 nm sh., 360 nm , 430 nm. . ^{1}H- NMR Spectral Data (DMSO-d_6) δ (ppm): 3.85 (s, OCH3), 6.2 (d, *J*=2.5 Hz,H-6), 6.45 (d, *J*=2.5 Hz, H-8), 6.95 (*d*, *J* = 8.0 Hz, H-5'), 7.5 (dd, *J*=2.5 & 7.5 Hz, H-6'), 7.85 (s, H- 2').

Results and Discussion:

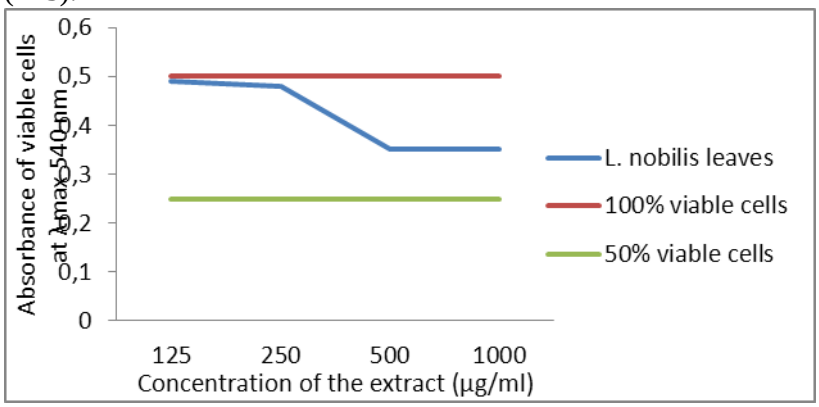
Hepatotoxicity:

The viability assay was applied with a broad range of concentrations of the studied extract of *L. nobilis* leaves (from 125-1000 µg/mL) on monolayer of rat hepatocytes. It revealed that the extract exerts no toxic effect on the monolayer hepatocyte layer, Table (1) and Figure (1).

Table (1): Hepatotoxicity of different concentrations of *L. nobilis* leaf extract.

Sample No.	Concentration µg/mL	Absorbance at (540 λ_{max}) of Neutral red for Viable Cells	Mean	S.D
L. nobilis leaves	125	0.436	0.49	0.07
		0.564		
		0.466		
	250	0.479	0.38	0.10
		0.382		
		0.277		
	500	0.307	0.32	0.05
		0.371		
		0.275		
	1000	0.340	0.28	0.07
		0.196		
		0.293		
Control	100% Viable cells	0.604	0.50	0.06
		0.502		
		0.495		
		0.438		
		0.478		
		0.496		

Figure (1): Viability of monolayer of rat hepatocytes after 2 hrs treatment with different concentrations of the Extract using NR Colourimetric Assay. Each point represents the Mean ± S.D (n=3).



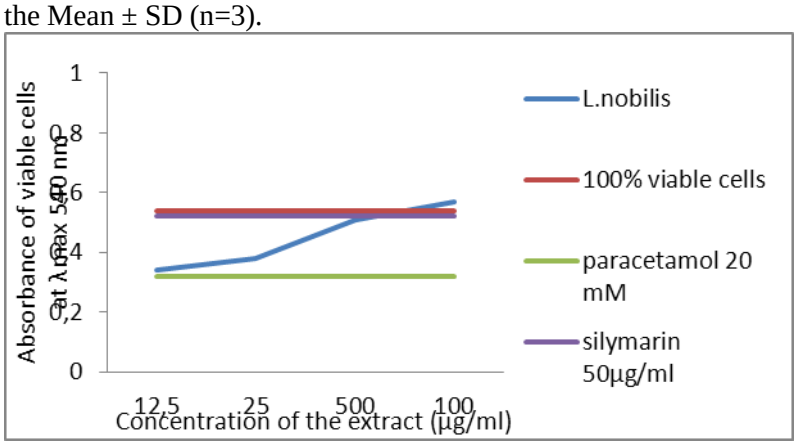
Evaluation of Hepatoprotective Activity Applying Rat Hepatocyte Monolayer:

The hepatoprotective effect of the tested extract against paracetamol toxic effect could be concluded from Table (2) and Figure (2). The *L. nobilis* leaves extract exhibited a hepatoprotective activity at 40 µg/ ml.

Table (2): Hepatoprotection of different concentrations of *L.nobilis* leaves under Investigation.

Sample No.	Concentration µg/mL	Absorbance at (540 λmax) of Neutral red for Viable Cells	Mean	S.D
L. nobilis leaves	12.5	0.371	0.34	0.04
		0.293		
		0.360		
	25	0.380	0.38	0.06
		0.321		
		0.443		
	50	0.513	0.51	0.05
		0.548		
		0.457		
	100	0.667	0.57	0.12
		0.610		
		0.443		
Control	100% viable cells	0.563	0.54	0.03
		0.556		
		0.507		
	Paracetamol 20 mM	0.363	0.32	0.03
		0.300		
		0.309		
	Silymarin 50 µg/mL	0.582	0.52	0.06
		0.516		
		0.470		

Figure (2): Viability of Monolayer of Rat Hepatocyte after 2 hrs Treatment with Different Concentrations of the Extract Followed by Treatment with 20 mM Paracetamol for 18 hrs. in comparison with 50 µg Silymarin as Control Using N.R Colourimetric Assay. Each Point Represents the Mean ± SD (n=3).



Identification and Structure Elucidation:

Following column chromatographic fractionation of the *L.nobilis* leaf extract, 14 compounds (**1–14**) were isolated and identified using conventional and spectral analyses mainly NMR spectroscopy and mass spectrometry.

Chromatographic behavior, UV spectral, ESI-MS (negative mode), ^{1}H & ^{13}C NMR data were consistent with those previously reported for, quercetin 3-*O*-α-rhamnopyranoside***1** (Babaei H. *et al*., 2008), kaempferol 3-*O*-α- rhamnopyranoside **2** (Babaei H. *et al*., 2008), quercetin 3-O- β-glucopyranoside **3** (Kang *et al*., 2002), Kaempferol 3-O- β- glucopyranoside***4** (Makuch M. and Awska I., 2011), Quercetin 3'-O- glucoside***5** (Susan and Paul, 1984), quercetin 3-O- β-galactopyranoside***6** (Mahmoud *et al*. 2002) , isorhamnetin 3-O- β- glucopyranoside*7 (Olennikov *et al*. 2011), isorhamnetin 3-O- β- galactopyranoside***8** (Sikorska M. and Matlawska I., 2001), quercetin 3-O- rutinoside***9** (Harborne J.B. 1994), kaempferol 3-O- rutinoside***10** (Brochado *et al*. 2003, Cardosa *et al*., 2005), isorhamnetin 3-O- rutinoside***11** (Harput *et al*., 2004), isorhamnetin***12** (Cao X. *et al*.,2009), quercetin **13** (Harborne, J.B., 1994) and kaempferol **14** (Smolarz, HD. , 2002).

*Compounds isolated for the first time from *L. nobilis* leaves.

Conclusion:

L. nobilis leaves proved its capability on synthesizing and accumulating an appreciable number of flavonoid glycosides in its leaves. The extract of these leaves showed hepatoprotective activity against paracetamol toxic effects on rat hepatocytes at low concentration of 40 µg / ml. These findings require further investigation to isolate and identify the active compounds and to unravel the underlying mechanisms of action.

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