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RESEARCH ARTICLE

In vitro hepatoprotective and antioxidant activities of crude extract and isolated compounds from *Ficus gnaphalocarpa*

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Abstract The in vitro hepatoprotective effect of the methanolic extract from *Ficus gnaphalocarpa* (Miq.) Steud. ex A. Rich (Moraceae) on the CCl_4-induced liver cell damage as well as the possible antioxidant mechanisms involved in this protective effect, were investigated. The phytochemical investigation of this methanolic extract led to the isolation of six compounds identified as: betulinic acid (1); 3-methoxyquercetin (2); catechin (3); epicatechin (4); quercetin (5); and quercitrin (6). The hepatoprotective activity of these compounds was tested in vitro against CCl_4-induced damage in rat hepatoma cells. In addition, radical-scavenging activity, β-carotene-linoleic acid model system, ferric-reducing antioxidant parameter and microsomal lipid peroxidation assays were used to measure antioxidant activity of crude extract and isolated compounds. Silymarin and trolox were used as standard references and, respectively, exhibited significant hepatoprotective and antioxidant activities. (5), (6) and (2) showed significant antioxidant and hepatoprotective activities as indicated by their ability to prevent liver cell death and lactate dehydrogenase leakage during CCl_4 intoxication. These results suggest that the protective effects of crude extract of *F. gnaphalocarpa* against the CCl_4-induced hepatotoxicity possibly involve the antioxidant effect of these compounds.

Keywords *Ficus gnaphalocarpa* · Hepatoprotective effect · Antioxidant activity · Carbon tetrachloride · Free radicals · Plant compounds · Silymarin

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Introduction

Increasing evidence suggests that oxidative stress-induced biochemical changes are crucial etiological factors in several chronic human diseases such as hepatitis, diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in aging process (Hogg 1998; Guyton et al. 1997). Based on growing interest in free radical biology and lack of effective therapies for most chronic diseases, the usefulness of antioxidants in protection against these diseases is warranted (Rubinstein 1962; Jacob and

Sotoudeh 2002; Avijeet et al. 2008; Yi-Hang et al. 2009; Huang et al. 2010).

Ficus gnaphalocarpa (Miq.) Steud. ex A. Rich (Moraceae) is a sub species of *Ficus sycomorus*, which takes the form of tree or shrub reach 10–30 m high. It has often stocky, up to 3 m in diameter, straight, cylindrical and slightly wide at the base. The crown is wide open, with spreading branches (Maydell 1983). The fruits are figs solitary or in pairs in the axils of leaves. They are 2–6 cm in diameter, more or less hairy, yellow or reddish when ripe. Fruiting takes place early in the dry season and rainy season (Cusset 1985). The bark of the trunk is smooth and scaly in places. It abounds in white or yellow latex (Maydell 1983).

Ethnopharmacological investigations show that *F. gnaphalocarpa* is widely used in treatment of many diseases such as hepatitis, jaundice (Maydell 1983), sickle-cell disease (Nongonierma et al. 2005), malaria (Asase et al. 2005) dysenteria, cholera, hemorrhagic diarrhea, gingivitis (Kerharo and Adam 1974), cough, throat inflammation, tuberculosis and some urogenital infections (Adjanohoun et al. 1989). This plant is also used as a tonic, stimulant, diuretic and in cases of difficult childbirth (Adjanohoun et al. 1989). In the present study, the effects of the crude extract of *F. gnaphalocarpa* and some isolated metabolites were examined for their in vitro hepatoprotective and antioxidant properties.

Materials and methods

Plant material

The stem bark of *F. gnaphalocarpa* was collected in January 2006 in Touloum (North region of Cameroon). The botanical identification of the plant was carried out at the Cameroon National Herbarium, where the voucher specimen was conserved under the reference number 28054/HNC.

Extraction and isolation of compounds

Air-dried and powdered stem bark samples of *F. gnaphalocarpa* (2 kg) were macerated in methanol (5 L) for 48 h at room temperature. The macerate was then passed through Whatman no. 1 filter paper. The filtrate was concentrated under vacuum into a paste to give a crude extract (300 g). This extract was subjected to a differential solubility on ethyl acetate to yield 60 g of ethyl acetate extract (FGEA). This extract was subjected to silica gel 60 (0.063–0200 mm) column chromatography using hexane/ethyl acetate and ethyl acetate/methanol gradients as eluents at a flow rate of 2 mL/min. Fractions (150 mL each) were collected as follows: pure hexane (fractions 1–5), hexane/ethyl acetate 95/5 (fractions 6–12), hexane/ethyl acetate 90/10 (fractions 13–25), hexane/ethyl acetate 85/15 (fractions 26–33), hexane/ethyl acetate 80/20 (fractions 34–41), hexane/ethyl acetate 70/30 (fractions 42–56), hexane/ethyl acetate 60/40 (fractions 57–70), hexane/ethyl acetate 50/50 (fractions 71–90), hexane/ethyl acetate 75/25 (fractions 91–102), ethyl acetate (fractions 103–115), ethyl acetate/methanol 90/10 (fractions 116–125) and methanol (fractions 126–132). These fractions were pooled on the basis of the thin layer chromatography analysis on five sub-fractions namely F_1 (fractions 1–5), F_2 (fractions 6–35), F_3 (fractions 36–69), F_4 (fractions 70–118) and F_5 (fractions 119–132) in the quantities of 7, 18, 11, 9 and 13 g, respectively. Two sub-fractions (F_2 and F_3) were separately subjected to silica gel 60 (0.063–0200 mm) column chromatography and preparative TLC using hexane/ethyl acetate gradients as eluent while dichloromethane/methanol gradients eluent were used to purify sub-fractions F_4 and F_5. Fractions of 50 mL each were collected. Purification of sub-fraction F_3 yields two compounds:

Betulinic acid ($C_{30}H_{48}O_3$) [compound (**1**); in hexane/ethyl acetate 80/20; white powder; 153 mg; m.p. 316–318°C; Rf: 0.5 (in hexane/ethyl acetate 85/15)] Exact Mass (HR EIMS) *m/z* 456.1 $[M]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) d (ppm): 4.66 and 4,53 (2H, d, $J = 19.8$ Hz, H-29), 3.09 (m), 2.17 (q), 1.62 (s, 3H, H-30), 0.68–91 (s, other CH_3); ^{13}C NMR: (75 MHz, $CDCl_3$) d (ppm) (C-1-C-30): 38.9, 27.1, 78.9, 38.4, 55.5, 18.4, 34.5, 40.8, 50.7, 36.9, 20.9, 25.7, 38.4, 42.6, 29.8, 32.4, 55.5, 49.4, 49.3, 150.3, 30.7, 37.2, 27.9, 15.4, 16.1, 15.9, 14.7, 179.1, 109.5, 19.3.

3-Methoxyquercetin ($C_{16}H_{12}O_7$) [compound (**2**); in dichloromethane/methanol 95/5; light yellow powder; 26 mg; m.p. 340–341°C; Rf: 0.6 (in dichloromethane/methanol 90/10)]; Exact Mass (HR ESIMS) *m/z* 317.1 $[M+H]^+$; ^{1}H-NMR (CD_6O, 600 MHz) δ: 7.70 (1H, d, $J = 1.8$ Hz, H-2′), 7.58 (1H, dd, $J = 1.8$ and 8.4 Hz, H-6′), 7.00 (1H, d, $J = 8.4$ Hz, H-5′), 6.50 (1H, d, $J = 1.8$, H-8), 6.26 (1H, d, $J = 1.8$ Hz, H-6), 3.87 (3H, s, OCH_3); ^{13}C-NMR (CD_6O, 150 MHz) δ: 178.6 (C-4), 164.0 (C-7), 162.3 (C-5), 156.9 (C-9), 148.3 (C-4′), 155.9(C-2), 145.0 (C-3′), 138.3 (C-3), 122.1 (C-1′), 121.2 (C-6′), 115.4 (C-5′), 115.3 (C-2′), 104.9 (C-10), 98.5 (C-6), 93.5 (C-8), 59.2 (3-OCH_3).

Purification of sub-fraction F_4 afforded three compounds

Catechin ($C_{15}H_{14}O_6$) [compound (**3**); in dichloromethane/methanol 85/15; pale yellow powder; 52 mg; m.p. 240–242°C; Exact Mass (HR EIMS) *m/z* 290.0 $[M]^+$; Rf: 0.5 (in dichloromethane/methanol 80/20)]; ^{1}H-NMR (acetone-d_6, 300 MHz): δ 2.54 (1H, dd, $J = 8.1$, 16.1 Hz, H-4), 2.98 (1H, dd, $J = 5.4$, 16.1 Hz, H-4), 4.01 (1H, m, H-3), 4.58 (1H, d, $J = 7.4$ Hz, H-2), 5.95 (1H, d,

$J = 2.1$ Hz, H-6), 5.88 (1H, d, $J = 2.1$ Hz, H-8), 6.76 (1H, dd, $J = 1.8, 8.1$ Hz, H-6′), 6.81 (1H, d, $J = 8.1$ Hz, H-5′), 6.91 (1H, d, $J = 1.8$ Hz, H-2′). ^{13}C-NMR (acetone-d_6, 150 MHz): δ 28.3 (C-4), 67.9 (C-3), 82.2 (C-2), 95.0 (C-8), 95.7 (C-6), 100.2 (C-10), 114.8 (C-2′), 115.3 (C-5′), 119.6 (C-6′), 131.7 (C-1′), 145.2 (C-3′),145.1 (C-4′), 156.4 (C-9), 156.1 (C-5), 157.2 (C-7).

Epicatechin ($C_{15}H_{14}O_6$) [compound (**4**); in dichloromethane/methanol 85/15; yellow powder; 15 mg; m.p. 195°C; Exact Mass (HR EIMS) *m/z* 290.0 [M]$^+$; Rf: 0.5 (in dichloromethane/methanol 85/15)] ^{1}H-NMR (acetone-d_6, 300 MHz): δ 2.54 (1H, dd, $J = 3.5, 16.7$ Hz, H-4), 2.96 (1H, dd, $J = 4.6, 16.7$ Hz, H-4), 4.01 (1H, m, H-3), 4.82 (1H, s, H-2), 5.95 (1H, d, $J = 2.3$ Hz, H-6), 5.88 (1H, d, $J = 2.1$ Hz, H-8), 6.76 (1H, dd, $J = 1.8, 8.1$ Hz, H-6′), 6.81 (1H, d, $J = 8.1$ Hz, H-5′), 6.91 (1H, d, $J = 1.8$ Hz, H-2′). ^{13}C-NMR (acetone-d_6, 150 MHz): δ 28.3 (C-4), 67.9 (C-3), 82.2 (C-2), 95.0 (C-8), 95.7 (C-6), 100.2 (C-10), 114.8 (C-2′), 115.3 (C-5′), 119.6 (C-6′), 131.7 (C-1′), 145.2 (C-3′),145.1 (C-4′), 156.4 (C-9), 156.1 (C-5), 157.2 (C-7).

Quercetin ($C_{15}H_{10}O_7$) [compound (**5**); in dichloromethane/methanol 90/10; yellow powder; 22 mg; m.p. 316°C; M_W: 302; Rf: 0.5 (in dichloromethane/methanol 85/15)]; ^{1}H-NMR (CD_6O, 600 MHz) δ: 7.84 (1H, d, 2.1, H-2′), 7.70 (1H, dd, $J = 2.1$ and 8.5 Hz, H-6′), 7.00 (1H, d, $J = 8.5$ Hz, H-5′), 6.53 (1H, d, $J = 1.9$ Hz, H-8), 6.27 (1H, d, $J = 1.9$ Hz, H-6); ^{13}C-NMR (CD_6O, 150 MHz) δ: 176.7 (C-4), 165.2 (C-7), 157.7 (C-5), 165.2 (C-9), 147.4 (C-4′), 148.5 (C-2), 146.0 (C-3′), 136.8 (C-3), 123.6 (C-1′), 121.4 (C-6′), 116.1 (C-5′), 115.7 (C-2′), 104.1 (C-10), 99.0 (C-6), 94.4 (C-8).

Purification of sub-fraction F_5 afforded one compound

Quercitrin ($C_{21}H_{20}O_{11}$) [compound (**6**); in dichloromethane/methanol 80/20; yellow crystal; 11 mg; m.p. 184–185°C; Exact Mass (HR ESIMS) *m/z* 471.0 [M+Na]$^+$ Rf: 0.4 (in dichloromethane/methanol 80/20)]; ^{1}H-NMR: (CD_3OD, 600 MHz) δ: 7.35 (1H, d, $J = 2.1$ Hz, H-2′), 7.32 (1H, dd, $J = 8.3, 2.1$ Hz, H-6′), 6.92 (1H, d, $J = 8.3$, H-5′), 6.38 (d, $J = 2.2$ Hz, H-8), 6.22 (1H, $J = 2.2$, d, H-6), 5.37 (d, $J = 1.7$ Hz, H-1″), 4.23 (1H, dd, $J = 3.4$ Hz, H-2″), 3.78 (1H, dd, $J = 9.3$ Hz, H-3″), 3.42 (1H, dq, $J = 9.6$ Hz, $J = 6.0$ Hz, H-5″), 3.38 (1H, dd, H-4″), 1.26 (3H, d, H 6″). ^{13}C-NMR (CD_3OD, 150 MHz), δ: 157.9 (C2), 134.8 (C3), 178.3 (C4), 157.1 (C5), 93.3 (C6), 164.4 (C7), 98.4 (C8), 163.3 (C9), 104.5 (C10), 121.6 (C1′), 114.9 (C2′), 148.3 (C3′), 145.0 (C4′), 115.5 (C5′), 122.7 (C6′), 102.1 (C1″), 71.9 (C2″), 70.7 (C3″), 70.6 (C4″), 70.5 (C5″), 17.7 (C6″).

For the screening of hepatoprotective and antioxidant activities, each of these isolated compounds was tested at the final concentrations of 12.5, 25, 50, 100 and 200 μg/mL, respectively, equivalent to 0.027, 0.054, 0.109, 0.219 and 0.438 mM for compound (**1**); 0.039, 0.079, 0.158, 0.316 and 0.632 mM for compound (**2**); 0.043, 0.083, 0.172, 0.344 and 0.689 mM for compounds (**3**) and (**4**); 0.039, 0.082, 0.165, 0.331 and 0.662 mM for compound (**5**); 0.027, 0.055, 0.111, 0.223 and 0.446 mM for compound (**6**).

Experimental design

Chemicals

All reagents used in the study were obtained from SIGMA Chemicals Co. (Dorset, UK) and Prolabo (Paris, France).

Screening for hepatoprotective activity using hepatoma cells

Hepatoma cell culture The effect of isolated compounds and crude extract from *F. gnaphalocarpa* on cell viability was assessed in a cell culture system using cells from the rat Morris hepatoma cell line BS TCL 41. The antihepatotoxic effects of the methanolic extract and isolated compounds were assayed on CCl_4-treated cells. The CCl_4 concentration used for cell culture treatment was previously determined and chosen because of its ability of induce up to 75% cell culture mortality (Chen et al. 1996; Rodeiro et al. 2008; Donfack et al. 2010). The methanolic extract and isolated compounds from *F. gnaphalocarpa* were not completely soluble in aqueous medium and had to be emulsified in dimethyl sulfoxide (DMSO) prior to their addition to the cell culture medium. A 3% solution of DMSO was used in cell culture medium. The cells were grown in Ham's F. 10/F10 medium supplemented with 20% (V/V) inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin 100× solution to prevent microbial contamination. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cell medium was changed twice a week. At 70–80% confluence, cells were trypsinized and seeded on 96-well plates at a cell density of 30,000 cells/well in serum-free culture medium. Twenty-four hours after cell seeding, cells were simultaneously exposed to 12.5, 25, 50, 100 and 200 μg/mL of the plant extracts and isolated compounds and 2.5 mM CCl_4 in fresh serum-free medium. The positive control was a set of cells maintained in a 2.5 mM CCl_4 test compounds free culture medium; while the negative control was a set of cells maintained in DMSO culture medium.

MTT assay Twenty-four hours after cell seeding, cells were treated with CCl_4 (2.5 mM) and incubated for 24 h at 37°C in the presence of 12.5, 25, 50, 100 and 200 μg/mL of crude extract or isolated compounds. Following removal of

the supernatants from each well, cells were washed with phosphate-buffered saline solution and incubated with 0.05% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in culture medium for 4 h. These supernatants were used to determine the lactate dehydrogenase (LDH) leakage. Subsequently, the medium was removed, and the cells washed with phosphate-buffered saline solution were incubated for 15 min in acidic isopropanol to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm using a multiwell plate reader. Viability was defined as the ratio (expressed as a percentage) of the absorbance of treated cells to that of untreated cells.

Lactate dehydrogenase assay For the LDH assay, 30,000 cells were seeded per well of 96 well plates in serum-free culture medium. Then, 24 h after cell seeding, cells were exposed to 2.5 mM CCl_4 and 12.5, 25, 50 100 or 200 µg/mL of the plant extract or isolated compounds. After 24 h of incubation, the supernatant was collected from each well. The LDH activity was measured in each supernatant using a cytotoxicity assay kit (Colorimetric Assay for Cytotoxicity Product NO. LK100 Oxford Biomedical Research), in accordance with the manufacturer's instructions. In this test, the intensity of the color obtained from reaction is proportional to the LDH activity. The absorbance was determined at 490 nm using a plate reader. The percentage of LDH released from the cells was determined as the ratio (expressed as a percentage) of the absorbance of treated cells to that of untreated cells.

Screening for antioxidant activity

Four model systems: 2,4-dinitrophenyl-1-picryl hydrazyl (DPPH) radical-scavenging activity, *β*-carotene-linoleic acid model system (*β*-CLAMS), ferric-reducing antioxidant power (FRAP) assay, and microsomal lipid peroxidation (IPL) were used to measure the antioxidant activities of the methanolic extract and isolated compounds from *F. gnaphalocarpa*. In each assay, the methanolic extract and isolated compounds were tested at doses of 12.5, 25, 50, 100 or 200 µg/mL. Trolox was used as a standard antioxidant. The EC_{50} values were then calculated for the crude extract and each individual compound.

Free radical-scavenging activity The free radical-scavenging activity of the methanolic extract and isolated compounds was evaluated by assessing the decrease in absorbance of 3,4-DPPH at 517 nm according to Brand-Williams et al. (1995). The decrease in absorbance was monitored at 517 nm, exactly 30 s after addition of the appropriate volume of the extract or methanol to the blank.

β-Carotene-linoleic acid model system assay The *β*-CLAMS method is based on the discoloration of *β*-carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at high temperature (Miller 1971). In brief, 1 mL *β*-carotene (0.02% w/v) dissolved in $CHCl_3$ was introduced in a 250 mL round-bottom flask. Linoleic acid (20 µL) and 200 mg Tween 20 were added to the mixture and the $CHCl_3$ was removed using a rotary evaporator. The 50 mL of distilled water was added and the flask was shaken vigorously until all the material dissolved. This test mixture was prepared fresh and used immediately. Three milliliters of test mixture plus 10 µL of compound solution or water (used as a blank) were added to each spectrophotometric cuvette. The spectrophotometric cuvettes were incubated at 50°C for 5 min and readings were carried out at 470 nm immediately after and then every 10 min for 3 h.

Ferric-reducing antioxidant power "FRAP" The FRAP assay measures the potential of antioxidants to reduce the Fe^{3+}/2,4,6-tripyridyl-*s*-triazine (TPTZ) complex present in stoichiometric excess to the blue colored Fe^{2+} form which increases the absorption at 593 nm. This method was carried out as described by Benzie and Strain (1996).

Lipid peroxidation assay Inhibition of lipid peroxidation was investigated using rat liver microsomes isolated by the calcium aggregation procedure as described by Garle and Fry (1989). Lipid peroxidation was non-enzymatically initiated using ascorbate as described by Ulf et al. (1989) and assayed for thiobarbituric acid-reactive substances (TBA-RS) were assayed according to Wills (1987).

Calculations and statistical analyses

Results are presented as mean ± SD. The total variation present in a set of data was analyzed by one-way analysis of variance (ANOVA) using the Graph Pad Prism software. A value of $P < 0.05$ was taken as statistically significant (*$P \leq 0.05$, **$P \leq 0.01$).

The EC_{50} values, taken as the concentrations of the sample required to scavenge 50% DPPH or to inhibit 50% of another oxidant mechanism, were estimated using Graph Pad Prism 3.0 software.

Results

Structures of compounds isolated from the methanolic extract

The structures of the isolated compounds and silymarin (used as an antihepatotoxic reference compound in this study) are shown in Fig. 1.

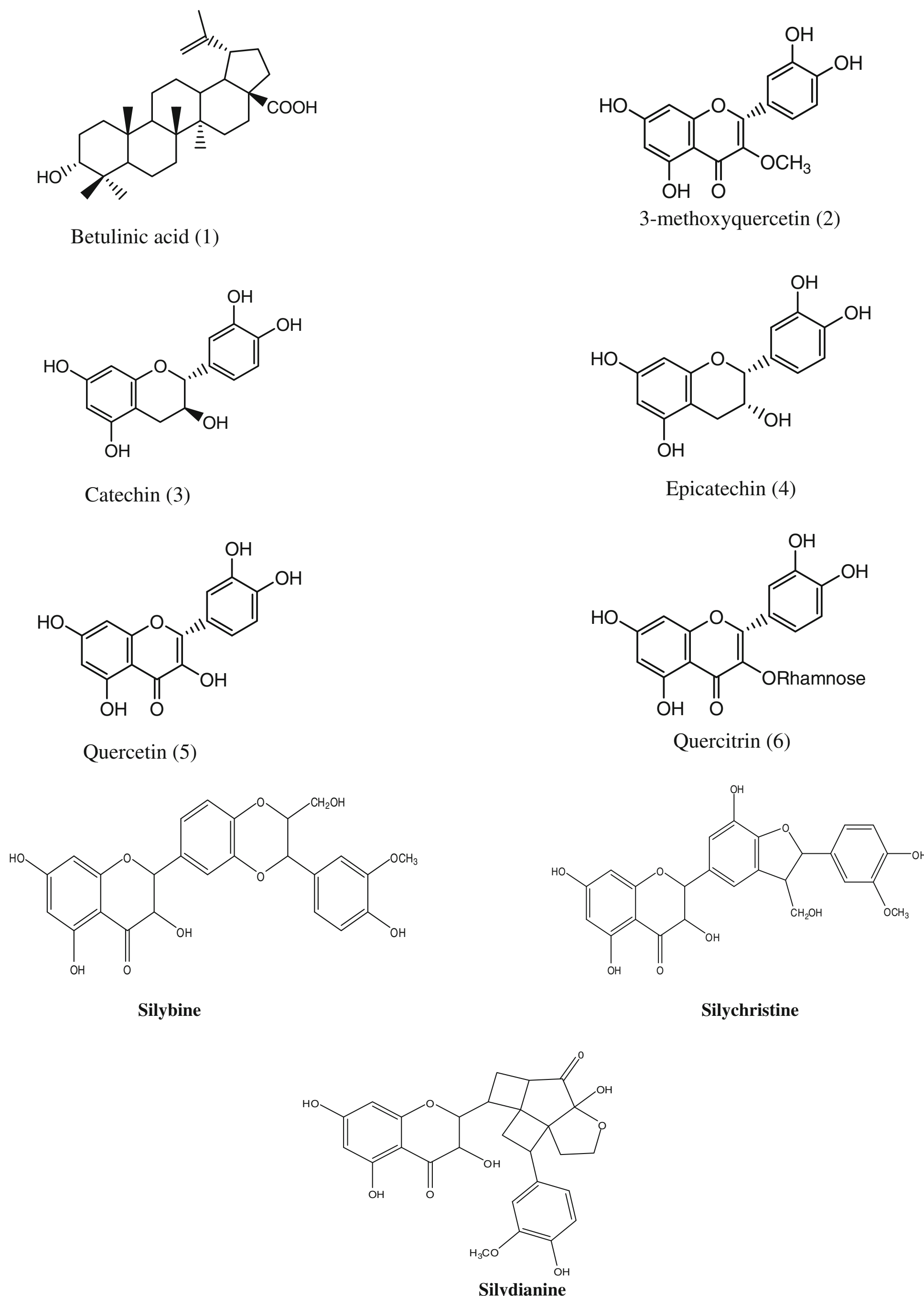

Fig. 1 Chemical structures of isolated compounds and silymarin (which is a mixing of silybine, silydianine and silychristine)

In vitro hepatoprotective activities of methanolic extract and isolated compounds

Metabolic activity can be evaluated by measuring the activity of the mitochondrial enzyme succinate dehydrogenase using the MTT test. MTT was designed for the quantification of both cell proliferation and cell viability in a cell population using a 96-well plate format. This test is widely used for the in vitro evaluation of bioactivity of plant extracts. In addition, membrane integrity can be evaluated by measuring LDH activity. LDH, an enzyme located in the cytoplasm, catalyses the interconversion of lactate and pyruvate. The presence of LDH in a medium can either be interpreted as a result of cell death or cell leakage. When cells are disrupted, LDH activity is increased. In the present study MTT and LDH assays were carried out to evaluate the in vitro hepatoprotective activity of the methanolic extract and isolated compounds. The results of these tests are summarized in Figs. 2 and 3. These results showed that until the dose of 100 μg/mL, cell viability increased with increasing amounts of the methanolic extract and isolated compounds. Quercetin (**5**),

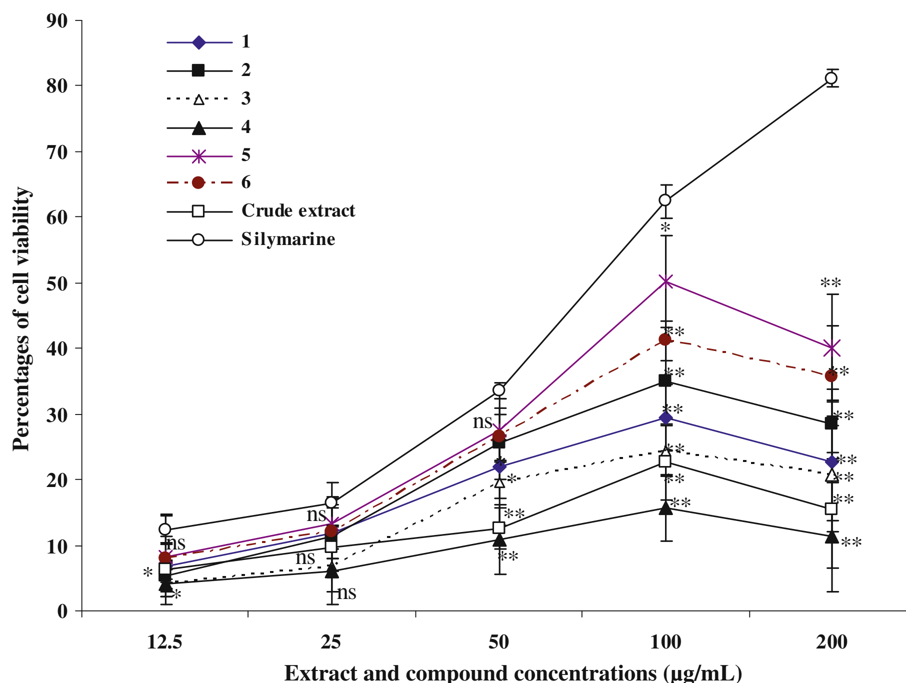

Fig. 2 MTT assay in rat hepatoma cells after a 24 h incubation with 12.5, 25, 50, 100 and 200 µg/mL extract and isolated compounds from *Ficus gnaphalocarpa*. The absorbance of the MTT formazan was determined at 570 nm in an ELISA/plate reader. Cell viability was defined as the ratio (expressed as a percentage) of the absorbance of treated cells to untreated cells. Reported values are the mean ± SD (standard deviation) of three independent experiments carried out in triplicate. (**1**)–(**6**): isolated compounds from *Ficus gnaphalocarpa*. **Significant, compared with reference compound $P < 0.01$. *Significant, compared with reference compound $P < 0.05$. [ns]Not significant, compared with reference compound ($P > 0.05$)

quercitrin (**6**) and 3-methoxyquercetin (**2**) exhibited the lowest percentage of LDH leakage. These compounds display an important hepatoprotective activity, which is characterized by their ability to preserve cell viability during CCl_4 intoxication.

Antioxidant activity of crude extract and isolated compounds

In order to determine the antioxidant effects of the methanolic extract and isolated compounds in terms of the mechanism of their hepatoprotective effects, the anti-lipid peroxidation in rat liver microsomes, the bleaching inhibition (measured by peroxidation of β-carotene), reducing power and scavenging activities were investigated. The results are shown in Fig. 4. These results showed that the most important antioxidant activities were obtained with quercetin (**5**), quercitrin (**6**) and 3-methoxyquercetin (**2**).

Discussion

There is increasing evidence that free radicals and reactive oxygen species (ROS) are generated by different kinds of chemicals such as food additives, industrial chemicals, pesticides and other undesirable contaminants in the air, food, water and soil to which humans are continuously exposed to. ROS and free radicals in general, play an important role in the etiology and pathogenesis of various diseases such as hepatitis, AIDS, hypertension and cardiovascular disorders. In recent years, many studies have been conducted on traditional medicines, in an attempt to develop new drugs for hepatitis (Liu 1989; Nandita et al. 2008; Ismail et al. 2009).

The toxicity induced by CCl_4 in vivo and in cultured hepatocytes, involves the stimulation of lipid peroxidation. CCl_4 treatment caused a significant decrease in cell viability. In addition, the toxin treatment initiated lipid peroxidation, caused leakage of enzymes like alanine transaminase and LDH levels (Gowri et al. 2008; Nandita et al. 2008; Rajesh et al. 2009). The most extensively studied aspect of free radical-induced liver injury is lipid peroxidation. CCl_4 is a well-known hepatotoxic agent and the preventive action of drugs on liver damage by CCl_4 has been widely used as an indicator of their liver protective activity. Changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Rubinstein 1962; Avijeet et al. 2008; Yi-Hang et al. 2009; Huang et al. 2010). Study of any herbal drug becomes more

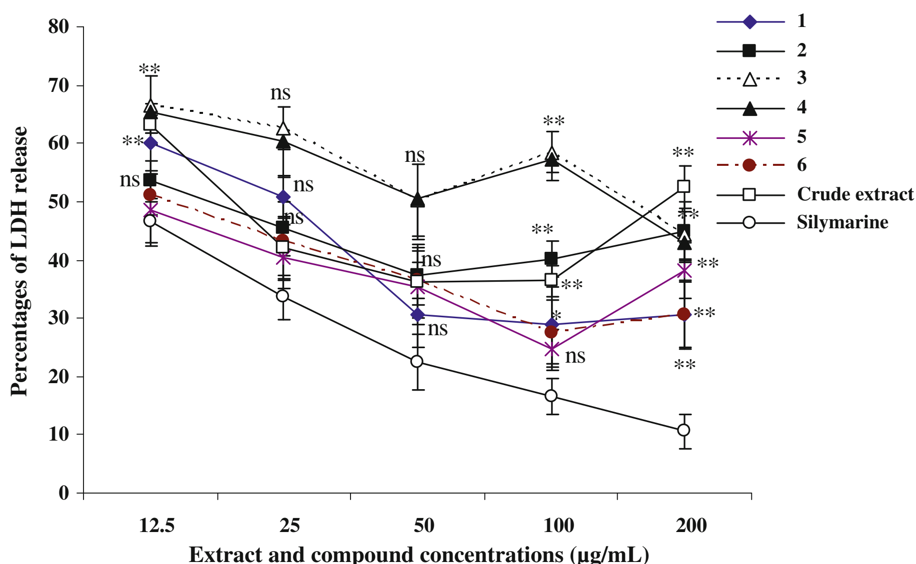

Fig. 3 LDH leakage from rat hepatoma cells after a 24 h incubation with 12.5, 25, 50, 100 and 200 µg/mL extract and isolated compounds from *Ficus gnaphalocarpa*. LDH activity was measured in the supernatants. Reported values are the mean ± SD (standard deviation) of three independent experiments carried out in triplicate. (**1**)–(**6**): isolated compounds from *Ficus gnaphalocarpa*. **Significant, compared with reference compound $P < 0.01$. *Significant, compared with reference compound $P < 0.05$. [ns]Not significant, compared with reference compound ($P > 0.05$)

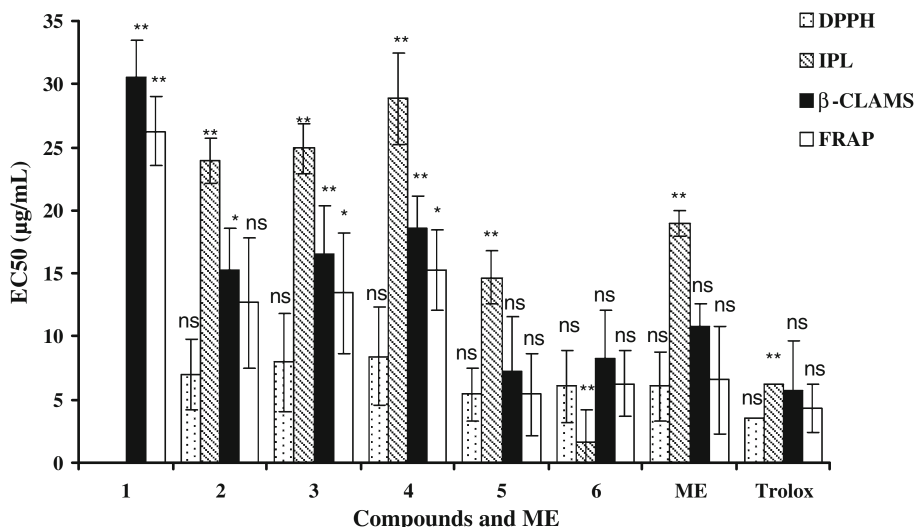

Fig. 4 Antioxidant activities of crude extract and isolated compounds from *Ficus gnaphalocarpa*. Values are EC_{50} ± SD of tree experiments in triplicate. *DPPH* radical-scavenging activity, *IPL* lipid peroxidation assay, *FRAP* ferric-reducing antioxidant power. *β*-CLAMS: inhibition of degradative oxidation of *β*-carotene. (**1**)–(**6**) are isolated compounds from *Ficus gnaphalocarpa*. **ME**: crude extract. **Trolox**: reference antioxidant compound. For each antioxidant mechanism, compounds were tested at five concentrations: 12.5, 25, 50, 100 and 200 µg/mL. **Significant, compared with reference compound $P < 0.01$. *Significant, compared with reference compound $P < 0.05$. [ns]Not significant, compared with reference compound ($P > 0.05$). Compound (**1**) shown no DPPH and IPL activities at the tested doses

significant when it ameliorates some diseases conditions (Anshu et al. 2008). The current research on plant-based medications focuses on isolation of biologically active substances from potent plants, their characterization and commercialization. Research in this direction has been greatly facilitated by modern physico-chemical techniques of isolation and structural elucidation (Suresh and Vandana 2008; Rajesh et al. 2009).

In the present study, we applied the MTT and LDH tests to evaluate the in vitro hepatoprotective activity of the

methanolic extract of *F. gnaphalocarpa* and isolated compounds in rat hepatoma cell cultures. The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of cells. In addition, the presence in the cell culture medium of the exclusively cytosolic enzyme LDH is indicative of cell membrane damage (Saad et al. 2003; Rajesh et al. 2009). We established that simultaneous treatment of hepatoma cells with the methanolic extract and isolated compounds from *F. gnaphalocarpa* prevented the toxic effects of CCl_4 as judged by cell viability and LDH leakage. The hepatoprotection, showed by the aptitude of these molecules to preserve cellular viability and to inhibit the leakage of LDH in extracellular medium was particularly pronounced with three isolated compound namely, quercetin (**5**), quercitrin (**6**) and 3-methoxyquercetin (**2**). Silymarin, which is an active constituent of the fruit of the milk thistle (*Silybum marianum*, Compositae) was used as standard reference and exhibited the best hepatoprotective activity against CCl_4-induced hepatotoxicity in rat Morris hepatoma cell.

Protective mechanisms relevant to the liver are of particular interest because free radicals and ROS play a central role in liver diseases pathology and progression. Dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Hensley et al. 2000; Higuchi and Gores 2003). Natural antioxidants may exert modulatory effects in cells through actions in antioxidant, drug metabolizing and repairing enzymes as well as working as signaling molecules in important cascades for cell survival (Nandita et al. 2008; Ning et al. 2008). Flavonoids are important phytochemicals that cannot be synthesized by humans. Quercetin is one of the ubiquitous flavonol-type flavonoids predominant in edible vegetables and fruits. As a member of the flavonol family, quercetin exhibited multiple biological effects on human health. It has been shown to prevent cardiovascular disease (Sesso et al. 2003), inhibit platelet aggregation and prevent atherosclerosis and thrombosis (Formica and Regelson 1995), have anticancer, anti-inflammatory responses, as well as antiulcer, antiallergic, and antiviral activities (Brown 1980; Middleton and Kandaswami 1992). Most of the pharmacological effects of quercetin are ascribed in part to its antioxidant activity. The antioxidant properties of quercetin might be due to its ability to chelate transition metal ions. Moreover, quercetin inhibited divalent cation-mediated lipid peroxidation, such as Fe^{2+} and Cu^{2+} (da Silva et al. 1998). Quercetin could reduce oxidative damage of macromolecules such as lipids and DNA (Negre-Salvayre and Salvayre 1992).

The fact that antioxidant agents inhibit carbon tetrachloride-induced liver damage (Muriel and Murelle 1990; Hussain et al. 2009; Naseem et al. 2009; Yi-Hang et al. 2009) prompted us to study the antioxidant effect of the isolated compounds. The DPPH radical-scavenging activity, β-CLAMS, FRAP assays, and microsomal lipid peroxidation are frequently used by researchers for a rapid evaluation of antioxidant activity (Benzie and Strain 1996; Gil et al. 2002). These systems were used in our study as well, with trolox as standard antioxidant compound. As shown by the results, all compounds tested in the present study exhibiting antioxidant activities. However, quercetin (**5**), quercitrin (**6**) and 3-methoxyquercetin (**2**) seemed to possess the highest antioxidant activities as indicated by their low EC_{50} values.

Conclusion

Taken together, our findings provide evidence that the methanolic extract and some isolated compounds from *F. gnaphalocarpa* exhibit hepatoprotective and antioxidant activities. This crude extract and isolated compounds might be useful for the prevention of toxic-induced and free radical-mediated liver diseases since it has been suggested that antioxidant compounds may be used as prophylactic agent.

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