

***Ficus cordata* Thunb (Moraceae) is a Potential Source of Some Hepatoprotective and Antioxidant Compounds**

¹Hubert J. Donfack, ²Rodrigue T. Kengap, ³B. Ngameni, ^{1,4}Pascal D.D. Chuisseu, ¹Angèle N. Tchana, ⁵Daniela Buonocore, ^{2,3}Bonaventure T. Ngadjui, ¹Paul F. Moundipa and ⁵Fulvio Marzatico

¹Department of Biochemistry, Laboratory of Pharmacology and Toxicology,

²Laboratory of Organic Chemistry, Department of Organic Chemistry,

Faculty of Science, University of Yaounde I, P.O. Box 812 Yaounde, Cameroon

³Department of Pharmacy and Traditional Pharmacotherapeutics,

Faculty of Medicine and Biomedical Science, University of Yaounde I, P.O. Box 1364 Yaounde, Cameroon

⁴Higher Institute of Health Sciences Université des Montagnes, P.O. Box 208, Bangangté, Cameroon

⁵Dipartimento di Medicina Legale, SC.Forensi e Farmacotossicologiche Sezione di Sc. Farmacologiche Tossicologiche via Ferrata 9-27100, Università Degli Studi di Pavia, Italy

Abstract: Background: Degenerative diseases in general and toxic hepatitis in particular remains a serious public health problem. The *in vitro* hepatoprotective effect of the crude extract and isolated compounds from *Ficus cordata* Thunb (Moraceae) on the CCl₄-induced liver cell damage as well as the possible antioxidant mechanisms involved in this protective effect, were investigated. The hepatoprotective activity of these compounds was tested *in vitro* against CCl₄-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. **Results:** The phytochemical investigation of this crude extract led to the isolation of five compounds identified as: β -amyrin acetate (1), Lupeol (2), Catechin (3), Epiafzelechin (4), Stigmasterol (5). These compounds showed significant hepatoprotective activities as indicated by their ability to prevent liver cell death and LDH leakage during CCl₄ intoxication. **Conclusion:** These results suggest that the protective effects of the crude extract of *Ficus cordata* against the CCl₄-induced hepatotoxicity possibly involve the antioxidant effect of some of these compounds.

Key words: *Ficus cordata*, hepatoprotective effect, carbon tetrachloride, antioxidant activity, plant compounds

INTRODUCTION

Lipid peroxidation has gained more importance today because of its involvement in pathogenesis of many diseases like atherosclerosis, cancer, hepatitis, diabetes mellitus, myocardial infarction and also ageing. Free radicals or Reactive Oxygen Species (ROS) are produced *in vivo* from various biochemical reactions and also from the respiratory chain as a result of occasional leakage. These free radicals are the main agents in lipid peroxidation. Antioxidants thus play an important role of protecting the human body against damage by reactive oxygen species (Lollinger, 1981; Cheeseman and

Scater, 2003). 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; Wu *et al.*, 2009; Huang *et al.*, 2010).

A number of *Ficus* species are used as food and for medicinal properties (Mousa *et al.*, 1994; De Amorin *et al.*, 1999; Lansky *et al.*, 2008). The biological activities of several plants of *Ficus* species have already been proven; some few examples include: *Ficus glabrata* (Hansson *et al.*, 1986), *Ficus racemosa* (Mandal *et al.*, 2000), *Ficus platyphylla* (Chindo *et al.*, 2003), *Ficus*

Corresponding Author: Dr. Donfack Jean Hubert, Department of Biochemistry Laboratory of Pharmacology and Toxicology, Faculty of Science, P.O. Box 812, University of Yaounde I, Cameroon
Tel: +23775133242, +237 22 10 15 81

bengalensis (Shukla *et al.*, 2004), *Ficus insipida* (Hansson *et al.*, 2005), *Ficus sycomorus* (Sandabe *et al.*, 2006), *Ficus asperifolia* (Annan and Houghton, 2008), *Ficus capensis* (Mpiana *et al.*, 2008), *Ficus glomerata* (Rao *et al.*, 2008), *Ficus bengalensis* (Singh *et al.*, 2009), *Ficus religiosa* (Singh and Goel, 2009), *Ficus exasperata* (Bafor and Igbinuwen, 2009), *Ficus hirta* (Li *et al.*, 2009), *Ficus chlamydocarpa* (Donfack *et al.*, 2010a), *Ficus gnaphalocarpa* (Donfack *et al.*, 2010b).

Ficus cordata Thunb (Moraceae) is a savana tree of around ten meters height found in Senegal, Angola, South Africa and Cameroon (Sabatie, 1985). The leaves of this plant are used against hyperaesthesia, ataxia, muscle tremor and padding motions and can kill heifers 48h after ingestion (Poumale *et al.*, 2008). Additional ethnopharmacological investigations showed that the stem bark of this plant is used by some western Cameroonian traditional healers for the treatment of *jaundice*; who can be a symptom of several related liver diseases.

In the present study, the effects of the crude extract of *Ficus cordata* and some isolated metabolites were examined for their *in vitro* hepatoprotective and antioxidant properties.

MATERIALS AND METHODS

Plant material: The stem bark of *Ficus cordata* was collected in March 2006 in the Center 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 8613/SRF/CAM.

Extraction and isolation of compounds: The powdered stem bark of *Ficus cordata* (1.8 kg) was extracted with CH₂Cl₂/MeOH (1:1) at room temperature for 24 h. After removing the solvents by evaporation under reduce pressure, the crude extract (CE; 155 g) was chromatographed on silica gel and eluted using hexane followed by hexane/ethyl acetate mixture; ethyl acetate and ethyl acetate/methanol gradients as eluent. Sixty fractions of 250 mL each were collected, then combined together on the basis of the analytic TLC in two fractions named A and B.

Fraction A (subfractions 1-15; 17 g) was chromatographed on silica gel and eluted with a mixture of hexane/ethyl acetate in increasing polarity to yield amount other β-amyrin acetate (1) (97 mg; m/z 426; amorphous powder; Rf: 0.80 using hexane-AcOEt: 19-1) and lupeol (2) (10 mg; m/z 426; m.p 214-215; Rf: 0,71 using hexane-

AcOEt: 19-1) (Swift, 1952; Thomson and Browsers, 1968). Fraction B (subfractions 30-60; 36 g) was chromatographed on silica gel and eluted with a mixture of CH₂Cl₂/MeOH of increasing polarity to yield, Catechin (3) (57 mg; m/z 290; m.p: 214; Rf: 0.36 using CH₂Cl₂/MeOH: 3-1) and Epiafzelechin (4) (60 mg; m/z 274; m.p: 252-254; Rf: 0,46 using CH₂Cl₂/MeOH: 3-1).

The powdered root of *Ficus cordata* (3 kg) was extracted with MeOH at room temperature for 48 h. The filtrate was then concentrated to give a crude extract (400 g). 150 g of the crude extract were chromatographed on silica gel and eluted using hexane followed by hexane/ethyl acetate of increasing polarity to yield β-amyrin acetate (1) (100 mg), lupeol (2) (10 mg) and stigmasterol (5) (20 mg; m/z 414; Rf: 0.34 using hexane-AcOEt: 17-3) (Kim *et al.*, 2005).

The structures of the isolated compounds were established using NMR spectral analysis such as 2D experiments, COSY, HMQC and HMBC and direct comparison with published information and with authentic specimens obtained by our research group. NMR spectra were measured on varian Unity 300 (300.125 MHz) and various Inova 500 (499.876 MHz) spectrometers. ESIMS was recorded on a Finnigan LCQ with a quaternary pump Rheos 4000 (Flux instrument). ESIHR mass spectra were recorded on Bruker FTICR 4.7 T mass spectrometer. EI-MS were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluor and kerosene as reference substance for HREI MS. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer from films. Flash chromatography was carried out on silica gel (230-400 mesh). Thin layer chromatography (TLC) was performed on silica gel 60 F254 (Merck) precoated aluminum sheets and spots were visualized using both UV light (254 and 366 nm) and 50% H₂SO₄ spray reagent.

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 (CE) from *Ficus cordata* 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 crude extract and isolated compounds were assayed on CCl₄-treated cells. The CCl₄ 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.*, 2010a; Donfack *et al.*, 2010b, c). The crude extract and isolated compounds from *Ficus cordata* were not completely soluble in aqueous medium and had to be emulsified in dimethylsulfoxide (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 100x solution to prevent microbial contamination. Cells were maintained in a humidified atmosphere with 5% CO₂ 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⁻¹ of the plant extracts and isolated compounds and 2.5 mM CCl₄ in fresh serum-free medium. The positive control was a set of cells maintained in a 2.5mM CCl₄ 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 CCl₄ (2.5 mM) and incubated with 12.5, 25, 50, 100 and 200 µg mL⁻¹ crude extract and isolated compounds for 24 hrs at 37°C. 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 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 (lactate dehydrogenase) assay, 30.000 cells were seeded per well of 96 well plates in serum-free culture medium. Then, 24th after cell seeding, cells were exposed to 2.5 mM CCl₄ and 12.5, 25, 50 100 or 200 µg mL⁻¹ of the plant extract or isolated compounds. After a 24 hrs 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 crude extract and isolated compounds from *Ficus cordata*. In each assay, the crude extract and isolated compounds were tested at doses of 12.5, 25, 50, 100 or 200 µg mL⁻¹. Trolox was used as a standard antioxidant. The EC₅₀ values were then calculated for the crude extract and each individual compound.

Free radical-scavenging activity: The free radical-scavenging activity of the crude 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 seconds after addition of the appropriate volume of the extract or methanol to the blank.

β-Carotene-linoleic acid model system (β-CLAMS) 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 CHCl₃ 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 CHCl₃ was removed using a rotary evaporator. The fifty milliliter 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 millilitres of test mixture plus 10 µL of extract/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 Ferric-Reducing Antioxidant Power (FRAP) assay measures the potential of antioxidants to reduce the Fe³⁺/2,4,6-

tripyrindyl-s-triazine (TPTZ) complex present in stoichiometric excess to the blue coloured 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 \pm SD. The total variation present in a

set of data was analysed by one-way analysis of variance (ANOVA) using the Graph Pad Prism software. A value of $p < 0.05$ was taken as statistically significant ($*p < 0.05$, $**p < 0.01$).

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.

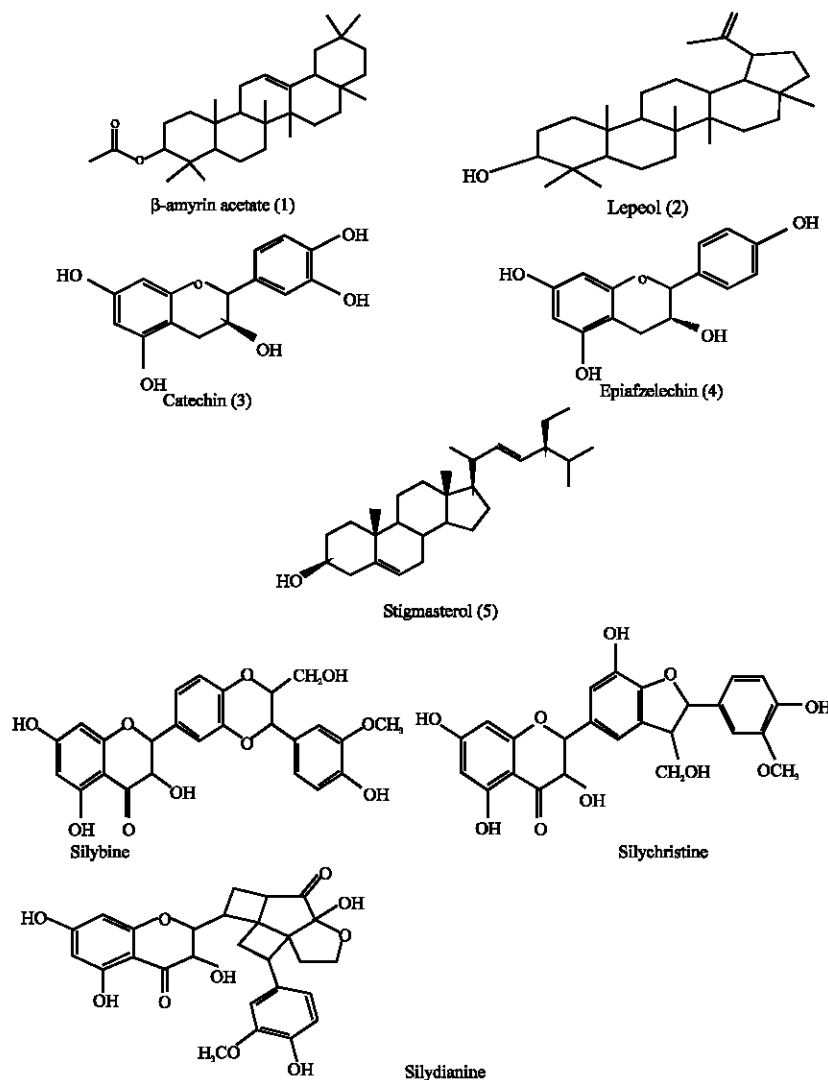


Fig. 1: Chemical structures of isolated compounds and silymarin (which is a mixing of silybine, silydianine and silychristine)

In vitro hepatoprotective activities of 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 lactate dehydrogenase (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 crude extract and isolated compounds. The results of these tests are summarized in Table 1 and 2. These results showed that until the dose of 100 $\mu\text{g mL}^{-1}$, cell viability increased with increasing amounts of the crude extract and isolated compounds. Lupeol (2), β -amyrin acetate (1) and Catechin (3) exhibited the lowest percentage of LDH leakage. These compounds display an important hepatoprotective activity, which was 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 crude 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 peroxydation of β -carotene), reducing power and scavenging activities were investigated. The results are shown in Table 3. These results show that apart of compound (1), all compounds showed antioxidant activities for the four studied mechanisms. But, the most important antioxidant activities were obtained with Catechin (3) Epiafzelechin (4) and Stigmasterol (5).

DISCUSSION

Free radicals and reactive oxygen species play a central role in liver diseases pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Hensely *et al.*, 2000; Higuchi and Gores, 2003). Natural antioxidants may act as protectors against several compounds but, more importantly, may exert modulatory effects in cells through actions in antioxidant, drug metabolizing and repairing enzymes as well as working as signalling molecules in important cascades for cell survival (Singh *et al.*, 2008; Wang *et al.*, 2008). In the past decade, the bioactivities of flavonoids on human health have given rise to much attention, especially the antioxidant activity. Flavonoids are important phytochemicals that cannot be synthesized by humans. Phenolics have been known to possess a capacity to scavenge free radicals. They are commonly found in both edible and nonedible plants and have multiple biological effects, including antioxidant activity (Káhkónen *et al.*, 1999; Valenzuela *et al.*, 2003). The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans *et al.*, 1996).

Study of any herbal drug becomes more significant when it ameliorates some diseases conditions. 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 (Naik and Panda, 2008; Krithika *et al.*, 2009). 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;

Table 1: MTT assay in rat hepatoma cells after a 24 h incubation with 12.5, 25, 50, 100 and 200 $\mu\text{g mL}^{-1}$ extract and isolated compounds from *Ficus cordata*

Compounds and crude extract	Concentration of compounds and percentages of cell viability				
	12.5 $\mu\text{g mL}^{-1}$	25 $\mu\text{g mL}^{-1}$	50 $\mu\text{g mL}^{-1}$	100 $\mu\text{g mL}^{-1}$	200 $\mu\text{g mL}^{-1}$
α -amyrin acetate (1)	5.59±2.15	9.28±1.89	28.89±3.78	36.02±1.27	37.23±4.48
Lupeol (2)	7.58±3.79	14.28±4.09	30.78±3.47	48.72±2.87	26.48±4.89
Catechin (3)	4.18±3.17	6.78±5.89	19.57±6.48	24.28±3.89	20.78±8.79
Epiafzelechin (4)	3.87±2.58	5.78±6.89	15.27±5.84	22.87±3.60	21.89±4.39
Stigmasterol (5)	3.12±3.79	4.47±5.89	9.18±4.89	19.18±5.78	16.48±8.01
Crude extract (CE)	4.74±2.54	7.05±2.27	26.75±3.07	34.81±3.17	33.78±2.78
Silymarine	12.35±2.25	16.30±3.28	33.50±1.25	62.39±2.54	81.12±1.35

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 deviations) of three independent experiments carried out in triplicate. (1) - (5): Isolated compounds from *Ficus cordata*, CE: Crude extract; Si: Silymarin taken as a hepatoprotective reference compound

Table 2: LDH leakage from rat hepatoma cells after a 24 h incubation with 12.5, 25, 50, 100 and 200 $\mu\text{g mL}^{-1}$ extract and isolated compounds from *Ficus cordata*

Compounds and ME	Concentration of compounds and LDH leakage from rat hepatoma cells				
	12.5 $\mu\text{g mL}^{-1}$	25 $\mu\text{g mL}^{-1}$	50 $\mu\text{g mL}^{-1}$	100 $\mu\text{g mL}^{-1}$	200 $\mu\text{g mL}^{-1}$
β -amyrin acetate (1)	60.45 \pm 3.58	57.89 \pm 5.48	48.26 \pm 2.81	31.25 \pm 5.19	40.18 \pm 6.71
Lupeol (2)	53.48 \pm 6.58	46.58 \pm 5.87	35.48 \pm 4.09	27.28 \pm 7.89	53.89 \pm 3.71
Catechin (3)	66.58 \pm 4.89	62.58 \pm 3.60	55.38 \pm 6.18	58.48 \pm 3.49	44.27 \pm 4.09
Epiafzelechin (4)	70.29 \pm 9.58	65.27 \pm 3.59	60.58 \pm 4.57	60.27 \pm 5.47	78.89 \pm 5.81
Stigmasterol (5)	78.57 \pm 3.08	70.07 \pm 4.75	37.61 \pm 6.28	64.48 \pm 5.31	88.18 \pm 6.72
Crude extract (CE)	40.80 \pm 4.70	34.80 \pm 3.47	22.89 \pm 6.89	30.47 \pm 5.78	63.80 \pm 5.71
Silymarine	46.70 \pm 3.89	33.60 \pm 3.76	22.50 \pm 4.75	16.60 \pm 3.04	10.60 \pm 2.97

LDH activity was measured in the supernatants. Reported values are the Mean \pm SD (standard deviations) of three independent experiments carried out in triplicate. (1) - (5): Isolated compounds from *Ficus cordata*, CE: Crude extract; Si: Silymarin taken as an antihepatotoxic reference compound

Table 3: Antioxidant activities of crude extract and isolated compounds from *Ficus cordata*. Values are EC₅₀ \pm SD of three experiments in triplicate

Compounds and ME	Biochemical antioxidant parameters (EC ₅₀ $\mu\text{g mL}^{-1}$)			
	DPPH	IPL	β -CLAMS	FRAP
β -amyrin acetate (1)	-	-	-	-
Lupeol (2)	23.48 \pm 4.48***	24.18 \pm 5.87***	22.47 \pm 4.57***	16.27 \pm 1.47*
Catechin (3)	8.01 \pm 2.78ns	24.89 \pm 1.78***	16.58 \pm 3.75***	13.47 \pm 4.79ns
Epiafzelechin (4)	11.67 \pm 2.45*	32.17 \pm 3.50***	18.47 \pm 5.43**	10.26 \pm 4.89ns
Stigmasterol (5)	17.28 \pm 3.24**	34.87 \pm 3.78***	27.89 \pm 1.27***	18.57 \pm 5.82**
Crude extract (CE)	17.23 \pm 3.87**	26.78 \pm 2.87***	37.13 \pm 2.90***	11.87 \pm 2.83ns
Trolox	3.52 \pm 2.74	6.19 \pm 1.04	5.74 \pm 3.89	4.37 \pm 1.89

DPPH: Radical scavenging activity; IPL: Lipid peroxidation assay; FRAP: ferric-reducing antioxidant power. β -CLAMS: Inhibition of degradative oxidation of β -carotene. (1) - (5) are isolated compounds from *Ficus cordata*. CE: crude extract. Trolox: reference antioxidant compound. For each antioxidant mechanism, compounds were tested at five concentrations: 12.5, 25, 50, 100 and 200 $\mu\text{g mL}^{-1}$. -: No activity was shown. ***Significant, compared with reference compound $p < 0.001$. **Significant, compared with reference compound $p < 0.01$. *Significant, compared with reference compound ($p < 0.05$). ^{ns}Not significant, compared with reference compound ($p > 0.05$)

Jain *et al.*, 2008; Wu *et al.*, 2009; Huang *et al.*, 2010). Carbon tetrachloride treatment initiated lipid peroxidation, caused leakage of enzymes like alanine transaminase and lactate dehydrogenase (LDH) levels (Singh *et al.*, 2008; Krithika *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 *Ficus cordata* 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. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide salt (MTT) to the red formazan derivative by mitochondrial succinate dehydrogenase in viable cells. In the lactate dehydrogenase (LDH) assay, leakage of the cytoplasmic located enzyme LDH into the extracellular medium is measured. The presence in the cell culture medium of the exclusively cytosolic enzyme LDH is indicative of cell membrane damage (Saad *et al.*, 2003; Krithika *et al.*, 2009). We established that simultaneous treatment of hepatoma cells with the crude extract and isolated compounds from *Ficus cordata* prevented the toxic effects of CCl₄ as judged by cellular 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 Lupeol (2), β -amyrin acetate (1) and Catechin (3). 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 CCl₄-induced hepatotoxicity in rat Morris hepatoma cell. 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 Fe²⁺ and Cu²⁺ (Da Silva *et al.*, 1998). Quercetin could reduce oxidative damage of macromolecules such as lipids and DNA (Negre-Salvayre and Salvayre, 1992).

It was demonstrated that Lupeol ameliorates aflatoxin B1-induced peroxidative hepatic damage in rats (Preetha *et al.*, 2006). Hepatoprotective effect of lupeol on tissue antioxidant defence system in cadmium-induced

hepatotoxicity in rats is known (Sunitha *et al.*, 2001). This pentacyclic triterpene also have some effect on the lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure (Nagaraj *et al.*, 2000). Lupeol also prevents free radical mediated macromolecular damage and alleviates benzoyl peroxide induced biochemical alterations in murine skin (Sultana *et al.*, 2003).

The fact that antioxidant agents inhibit carbon tetrachloride-induced liver damage (Muriel and Murelle, 1990; Zeashan *et al.*, 2009; Qureshi *et al.*, 2009; Wu *et al.*, 2009) prompted us to study the antioxidant effect of the crude extract and 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. These systems were used in our study as well, with trolox as standard antioxidant compound. As shown by the results, apart of compound (1), all compounds tested in the present study exhibit antioxidant activities.

This study provides a scientific basis for the ethnomedical claims that *Ficus cordata* is effective against certain liver-related diseases. Taken together, our findings provide evidence that the crude extract and some isolated compounds from *Ficus cordata* 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.

ACKNOWLEDGMENTS

This research was supported by the University Centre for International Cooperation and Development (CICOPS) and by the Institute for Right to University Studies (ISU University of Pavia, Italy). The authors sincerely thank Prof Paola Vita Finzi and Prof. Giovanni Vidari (University of Pavia- Italy) for their support. They also thank Dr. Kerr G. Philip Charles Sturt University Locked Bag 588 Wagga Wagga (Australia), for the critical reading of the manuscript.

REFERENCES

Annan, K. and P.J. Houghton, 2008. Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. and *Gossypium arboreum* L., wound-healing plants of Ghana. *J. Ethnopharmacol.*, 119: 141-144.

Bafor, E.E. and O. Igbinuwen, 2009. Acute toxicity studies of the leaf extract of *Ficus exasperata* on haematological parameters, body weight and body temperature. *J. Ethnopharmacol.*, 123: 302-307.

Benzie, I.F. and J.J. Strain, 1996. The Ferric Reducing Ability of Plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Anal. Biochem.*, 239: 70-76.

Brand-Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.*, 28: 25-30.

Brown, J.P., 1980. A review of the genetic effects of naturally occurring flavonoids, anthroquinones and related compounds. *Mutat. Res.*, 75: 243-277.

Cheeseman, K.H. and T.F. Scater, 2003. Free radical in medicine. *Br. Med. Bull.*, 49: 479-724.

Chen, S., S., Morimoto, M., Tamatani, K. Fukuo and T. Nakahashi *et al.*, 1996. Calcitonin prevents CCl₄-induced hydroperoxide generation and cytotoxicity possibly through C1b receptor in rat hepatocytes. *Biochem. Biophys. Res. Commun.*, 218: 865-871.

Chindo, B.A., S. Amos, A.A. Odotola, H.O. Vongtau, J. Abbah, C. Wambebe and K.S. Gamaniel, 2003. Central nervous system activity of the methanol extract of *Ficus platyphylla* stem bark. *J. Ethnopharmacol.*, 8: 131-137.

Da Silva, E.L., M.K. Piskula, N. Yamamoto, J.H. Moon and J. Terao, 1998. Quercetin metabolites inhibit copper ion-induced lipid peroxidation in rat plasma. *FEBS Lett.*, 430: 405-408.

De Amorin, A., R.B. Helcio, P.P. Jorge-Carauta, D. Lopes and M.A.C. Kaplan, 1999. Anthelmintic activity of the latex of *Fiscus* species. *J. Ethnopharmacol.*, 64: 225-258.

Donfack, J.H., C.C. Simo, B. Ngameni, A.N. Tchana and P.G. Kerr *et al.*, 2010a. Antihepatotoxic and antioxidant activities of methanol extract and isolated compounds from *Ficus chlamydocarpa*. *Nat. Prod. Commun.*, 5: 1607-1612.

Donfack, J.H., A. Dawe, N.T. Florence, K.D. Gilbert and T.N. Angele *et al.*, 2010b. *In vitro* hepatoprotective and antioxidant activities of crude extract and isolated compounds from *Ficus gnaphalocarpa*. *Inflammopharmacology*, 19: 35-43.

Donfack, J.H., G.W. Fotso, B. Ngameni, F.N. Tsofack and A. Tchoukoua *et al.*, 2010c. *In vitro* hepatoprotective and antioxidant activities of oleanolic and ursolic acids from *Irvingia gabonensis*. *Asian J. Trad. Med.*, 5: 79-88.

Formica, J.V. and W. Regelson, 1995. Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol.*, 33: 1061-1080.

Garle, M.J. and J.R. Fry, 1989. Detection of reactive metabolites *in vitro*. *Toxicology*, 154: 101-110.

Hansson, A., G. Veliz, C. Naquira, M. Amren, M. Arroyo and G. Arevalo, 1986. Preclinical and clinical studies with latex from *Ficus glabrata* hbk, a traditional intestinal anthelmintic in the amazonian area. *J. Ethnopharmacol.*, 17: 105-138.

- Hansson, A., J.C. Zelada and H.P. Noriega, 2005. Reevaluation of risks with the use of *Ficus insipida* latex as a traditional anthelmintic remedy in the Amazon. *J. Ethnopharmacol.*, 98: 251-257.
- Hensely, K., K.A. Robinson, S.P. Gobbita, S. Salsman and R.A. Floyd, 2000. Reactive oxygen species, cell signaling and cell injury. *Free Radic. Biol. Med.*, 28: 1456-1462.
- Higuchi, H. and G.J. Gores, 2003. Mechanisms of liver injury: An overview. *Curr. Mol. Med.*, 3: 483-490.
- Huang, B., X. Ban, J. He, J. Tong, J. Tian and Y. Wang, 2010. Hepatoprotective and antioxidant activity of ethanolic extracts of edible lotus (*Nelumbo nucifera* Gaertn.) leaves. *Food Chem.*, 120: 873-878.
- Jacob, R.A. and G. Sotoudeh, 2002. Vitamin C function and status in chronic disease. *Nutr. Clin. Care*, 5: 66-74.
- Jain, A., M. Soni, L. Deb, A. Jain, S.P. Rout, V.B. Gupta and K.L. Krishna, 2008. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. Leaves. *J. Ethnopharmacol.*, 115: 61-66.
- Kahkonen, M.P., A.I. Hopia, H.J. Vuorela, J.P. Rauha, K. Pihlaja, T.S. Kulaja and M. Heinonen, 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.*, 47: 3954-3962.
- Kim, D.K., D.P. Lim, J.W. Kim, H.W. Park and J.S. Eun, 2005. Antitumoral and antiinflammatory constituents from *Celtis sinensis*. *Arch. Pharm. Res.*, 28: 39-43.
- Krithika, R., R. Mohankumar, R.J. Verma, P.S. Shrivastav, I.L. Mohamad, P. Gunasekaran and S. Narasimhan, 2009. Isolation, characterization and antioxidative effect of phyllanthin against CCl₄-induced toxicity in HepG2 cell line. *Chem. Biol. Interact.*, 181: 351-358.
- Lansky, E.P., H.M. Paavilainen, A.D. Pawlus and R.A. Newman, 2008. *Ficus* spp. (fig): Ethnobotany and potential as anticancer and anti-inflammatory agents. *J. Ethnopharmacol.*, 119: 195-213.
- Li, Y., J. Duan, T. Guo, W. Xie and S. Yan *et al.*, 2009. *In vivo* pharmacokinetics comparisons of icariin, emodin and psoralen from Gan-kang granules and extracts of herba Epimedii, Nepal dock root, *Ficus hirta* yahl. *J. Ethnopharmacol.*, 124: 522-529.
- Lollinger, J., 1981. *Free Radicals and Food Additives*. Taylor and Francis, London, pp: 121.
- Mandal, S.C., T.K. Maity, J. Das, B.P. Saba and M. Pal, 2000. Anti-inflammatory evaluation of *Ficus racemosa* Linn. leaf extract. *J. Ethnopharmacol.*, 72: 87-92.
- Middleton, E. and C. Kandaswami, 1992. Effects of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol.*, 43: 1167-1179.
- Miller, H.E., 1971. A simplified method for the evaluation of anti-oxidant. *J. Am. Oil Chem. Soc.*, 48: 91-97.
- Mousa, O., P. Vuorela, J. Kiviranta, S.A. Wahab, R. Hiltunen and H. Vuorela, 1994. Bioactivity of certain Egyptian *Ficus* species. *J. Ethnopharmacol.*, 41: 71-76.
- Mpiana, P.T., V. Mudogo, D.S. Tshibangu, E.K. Kitwa and A.B. Kanangila *et al.*, 2008. Antisickling activity of anthocyanins from *Bombax pentadrum*, *Ficus capensis* and *Ziziphus mucronata*: Photodegradation effect. *J. Ethnopharmacol.*, 120: 413-418.
- Muriel, P. and M. Murelle, 1990. Prevention by silymarin of membrane alterations in acute CCl₄ liver damage. *J. Applied Toxicol.*, 10: 275-279.
- Nagaraj, M., S. Sunitha and P. Varalakshmi, 2000. Effect of lupeol, a pentacyclic triterpene, on lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure. *J. Applied Toxicol.*, 20: 413-417.
- Naik, S.R. and V.S. Panda, 2008. Hepatoprotective effect of ginkgoselect phytosome® in rifampicin induced liver injury in rats: Evidence of antioxidant activity. *Fitoterapia*, 79: 439-445.
- Nègre-Salvayre, A. and R. Salvayre, 1992. Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cell lines. *Free Radic. Biol. Med.*, 12: 101-106.
- Poumale, H.M.P., R.T. Kengap, J.C. Tchouankeu, F. Keumedjio, H. Laatsch and B.T. Ngadjui, 2008. Pentacyclic triterpenes and other constituents from *Ficus cordata* (Moraceae). *Z. Naturforsch.*, 63: 1335-1338.
- Preetha, S.P., M. Kannappan, E. Selvakumar, M. Nagaraj and P. Varalakshmi, 2006. Lupeol ameliorates aflatoxin B₁- induced peroxidative hepatic damage in rats. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, 143: 333-339.
- Qureshi, N.N., B.S. Kuchekar, N.A. Logade and M.A. Haleem, 2009. Antioxidant and hepatoprotective activity of *Cordia macleodii* leaves. *Saudi Pharm. J.*, 17: 299-302.
- Rao, C.V., A.R. Verma, M. Vijayakumar and S. Rastogi, 2008. Gastroprotective effect of standardized extract of *Ficus glomerata* fruit on experimental gastric ulcers in rats. *J. Ethnopharmacol.*, 115: 323-326.
- Rice-Evans, C.A., N.J. Miller and G. Paganga, 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.*, 20: 933-956.
- Rodeiro, I., M.T. Donato, I. Martinez, I. Hernandez and G. Garrido *et al.*, 2008. Potential hepatoprotective effects of new Cuban natural products in rat hepatocytes culture. *Toxicol. In vitro*, 22: 1242-1249.

- Rubinstein, D., 1962. Epinephrine release and liver glycogen levels after carbon tetrachloride administration. *Am. J. Physiol.*, 203: 1033-1037.
- Saad, B., G. Abu-Hijleh and U.W. Suter, 2003. Polymer Biocompatibility Assessment by Cell Culture Techniques. In: The PMB Series Volume 1: Introduction Polymeric Biomaterials, Arshady, R. (Ed.). The Citus Books, Australia.
- Sabatie, B., 1985. Flore du Cameroun, Moraceae (incl; Cecropiaceae) Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (MESRES) (Ed.), Yaounde-Cameroun, pp: 154-155.
- Sandabe, U.K., P.A. Onyeyili and G.A. Chibuzo, 2006. Phytochemical screening and effect of aqueous extract of *Ficus sycomorus* L. (Moraceae) stem bark on muscular activity in laboratory animals. *J. Ethnopharmacol.*, 103: 481-483.
- Sesso, H.D., J.M. Gaziano, S. Liu and J.E. Buring, 2003. Flavonoid intake and the risk of cardiovascular disease in women. *Am. J. Clin. Nutr.*, 77: 1400-1408.
- Shukla, R., S. Gupta, J.K. Gambhir, K.M. Paha and P.S. Murthy, 2004. Antioxidant effect of aqueous extract of the bark of *Ficus bengalensis* in hypercholesterolaemic rabbits. *J. Ethnopharmacol.*, 92: 47-55.
- Singh, D. and R.K. Goel, 2009. Anticonvulsant effect of *Ficus religiosa*: Role of serotonergic pathways. *J. Ethnopharmacol.*, 123: 330-334.
- Singh, N., V. Kamath, K. Narasimhamurthy and P.S. Rajini, 2008. Protective effect of potato peel extracts against carbon tetrachloride-induced liver injury in rats. *Environ. Toxicol. Pharmacol.*, 26: 241-246.
- Singh, R.K., S. Mehta, D. Jaiswal, P.K. Rai and G. Watal, 2009. Antidiabetic effect of *Ficus bengalensis* aerial roots in experimental animals. *J. Ethnopharmacol.*, 123: 110-114.
- Sultana, S., M. Saleem, S. Sharma and N. Khan, 2003. Lupeol, a triterpene, prevents free radical mediated macromolecular damage and alleviates benzoyl peroxide induced biochemical alterations in murine skin. *Indian J. Exp. Biol.*, 41: 827-831.
- Sunitha, S., M. Nagaraj and P. Varalakshmi, 2001. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. *Fitoterapia*, 72: 516-523.
- Swift, L.I., 1952. Isolation of β -sitosterol, β -sitosterol-3-O- β -D-glucopyranoside from the juice of Florida valencia oranges (*Citrus sinensis* L.). *J. Am. Chem. Soc.*, 74: 1099-1100.
- Thomson, M.J. and W.S. Browers, 1968. Lupeol and 30-norlupan-3 β -ol-20-one from the coating of the castor bean (*Ricinus communis* L.). *Phytochemistry*, 7: 845-847.
- Ulf, A.N., O. Lars-Inge, C. Gunnar and B.A. Christin, 1989. Inhibition of lipid peroxidation by spin labels. *J. Biol. Chem.*, 264: 11131-11135.
- Valenzuela, A., J. Sanhueza and S. Nieto, 2003. Cholesterol oxidation: Health hazard and the role of antioxidants in prevention. *Biol. Res.*, 36: 291-302.
- Wang, N., P. Li, Y. Wang, W. Peng and Z. Wu *et al.*, 2008. Hepatoprotective effect of *Hypericum japonicum* extract and its fractions. *J. Ethnopharmacol.*, 116: 1-6.
- Wills, E.O., 1987. Evaluation of Lipid Peroxidation and Biological Membrane. In: *Biochemical Toxicology*, Snell, K. and B. Mullock (Eds.). IRL Press, Oxford, UK., pp: 127-151.
- Wu, Y.H., X.M. Zhang, M.H. Hu, X.M. Wu and Y. Zhao, 2009. Effect of *Laggera alata* on hepatocyte damage induced by carbon tetrachloride *in vitro* and *in vivo*. *J. Ethnopharmacol.*, 126: 50-56.
- Zeashan, H., G. Amresh, S. Singh and C.V. Rao, 2009. Hepatoprotective and antioxidant activity of amaranthus spinosus against CCl₄ induced toxicity. *J. Ethnopharmacol.*, 125: 364-366.