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Antihepatotoxic and Antioxidant Activities of Methanol Extract and Isolated Compounds from *Ficus chlamydocarpa*

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Free radicals, in particular radical oxygen species (ROS), play an important role in the aetiology and pathogenesis of various diseases. Current research in many countries focuses on the use of local medicinal plants as a promising source of liver protective agents. This paper describes the hepatoprotective effects of the methanol extract and four isolated compounds from *Ficus chlamydocarpa* on CCl_4-induced liver damage, as well as the possible antioxidant mechanisms involved in this protection. The DPPH test, along with the ß-Carotene-Linoleic Acid Model System and Ferric-Reducing Antioxidant Power assays, as well as the inhibition of microsomal lipid peroxidation were used to measure radical-scavenging and antioxidant activities. Pretreatment of rats with the methanol extract of *F. chlamydocarpa* before CCl_4 administration, significantly prevented serum increase of hepatic enzyme markers, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), in a dose-dependent manner. The hepatoprotection was also associated with a significant enhancement in hepatic reduced glutathione (GSH) and a marked decrease of liver malondialdehyde (MDA). Among the four compounds **1**-**4**, isolated from the methanol extract, α-amyrin acetate (**1**) and luteolin (**4**) showed a significant hepatoprotective activity, as indicated by their ability to prevent liver cell death and lactate dehydrogenase (LDH) leakage during CCl_4 intoxication.

Keywords: *Ficus chlamydocarpa*, antihepatotoxic effect, antioxidant activity, free radicals, flavonoids.

Reactive oxygen species (ROS) are involved in the development of various ailments such as atherosclerosis, diabetes, cancer, AIDS, cardiovascular, Alzheimer's and other neurodegenerative diseases [1,2]. Hence ROS scavenger compounds can reduce the incidence of free radical-mediated disorders; and in this regard, there is a growing interest in dietary antioxidants such as vitamins E and C, carotenoids, and plant polyphenols for the prevention and cure of various ROS-related diseases [3].

Free radicals have been implicated in the pathogenesis of alcohol-induced liver injury in humans and CCl_4-induced liver injury in rats. On the other hand, exposure to high levels of environmental and food toxins, a possible source of ROS in tissues, can induce liver intoxication, due to the important role of this organ in body detoxification from xenobiotics. Therefore, a diet based on plants containing antioxidant substances could afford significant protection against hepatic injuries [4]. In this regard, growing attention has been given to plants used in traditional medicines, as novel remedies for treating hepatic disorders [4].

Table 1: Effects of the methanol extract of *F. chlamydocarpa* stem bark on MDA and GSH levels in rat liver homogenates, and GOT and GPT values in rat serum after CCl_4 administration..

Groups	MDA (µmol/mg of liver protein)	GSH (µmol/mg of liver protein)	GOT (IU/L)	GPT (IU/L)
Normal control	36.8±5.5**	27.0±4.3**	81,9±6.8**	37.9±4.9**
CCl_4 control	121.0±3.2	10.9±2.2	204.3±3.9	194.8±5.0
CCl_4 ME (25 mg/kg)	95.4±6.2**	11.5±3.17^{ns}	195.4±4,8*	104.0±3.87**
CCl_4 ME (50 mg/kg)	75.9±4.1**	14.4±6.5^{ns}	119.3±5.8**	78.8±7.67**
CCl_4 ME (100 mg/kg)	56.9±5.3.**	21.7±3.4**	89.0±5.6**	59.9±2.8**
CCl_4 ME (200 mg/kg)	86.8±6.3**	13.5±3.8^{ns}	106.7±3.5**	84.7±3.1**
Silymarin (100mg/mL)	42.6±3.4**	23.5±2.5*	57.7±2.87**	46.8±5.0**

Values are the mean ± SD of five rats per group. $^{**}P < 0.01$; $^{*}P < 0.05$ significantly different from the CCl_4-group. ME: methanol extract of *F. chlamydocarpa*. **ns**: no significant different from the CCl_4 group, $P > 0.05$.

Ficus chlamydocarpa (Moraceae) is traditionally used in Cameroon for the treatment of different diseases, from filaria to diarrheal infections and tuberculosis [5]. Another ethnopharmacological survey has revealed that a decoction of the stem bark is used in West Cameroon folk medicine for treating jaundice, which is commonly considered a symptom of liver-related diseases.

In the present study, the antihepatotoxic and antioxidant properties of the methanol extract of *F. chlamydocarpa* and four isolated metabolites, α-amyrin acetate (**1**), alpinumisoflavone (**2**), genistein (4',5,7-trihydroxyisoflavone) (**3**), and luteolin (3',4',5,7-tetrahydroxy flavone) (**4**), were examined in CCl_4-treated rats and in rat hepatoma cells.

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 [6]. As a matter of fact, changes associated with CCl_4-induced liver damage are similar to those of acute viral hepatitis in humans [7]. It is known that hepatic cytochrome P450 undergoes significant destruction during metabolism of CCl_4 [8]. Studies on hepatoprotective models show that CCl_4 is first metabolized to the highly reactive free radical $CCl_3^{\bullet}$ by cytochrome P450 in the endoplasmic reticulum of the liver [9]. In the presence of oxygen, this free radical promotes a cyclical non-enzymatic peroxidation of polyunsaturated fatty acid esters occurring in membrane phospholipids, leading to functional and morphological changes in cell membranes [10.11]. This parallels the consequences of lipid peroxidation in human liver due to free radical-induced injury. Moreover, the altered membrane permeability often results in leakage of large quantities of enzymes into the blood stream [12], causing, eventually, even massive necrosis of the liver [13]. Therefore, the ability to reduce the leakage of hepatic enzymes is evidence of the hepatoprotective action of a drug. In our model system, we measured the levels of GOT and GPT enzymes in rat serum; in particular, GPT has been shown to be a more sensitive test than GOT to assess hepatocellular damage [14]. We also measured the values of MDA and GSH in rat liver homogenates, since they have been reported to be sensitive indicators of liver injury [15]. In particular, MDA, the end product of lipid peroxidation, has been extensively studied and measured as an index of lipid peroxidation and as a biomarker of oxidative stress [16].

In the present study, severe liver damage was induced in rats treated with CCl_4, as indicated by a marked increase in liver MDA, and serum GOT and GPT values in comparison with control rats (Table 1). However, rats pretreated with the methanol extract of *F. chlamydocarpa* showed an evident reduction in these values, in a dose dependent manner, with a particular effect on reducing GPT leakage. In addition, the increased levels of hepatic GSH confirmed the significant hepatoprotective effect of the methanol extract, the dose of 100 mg/kg being the most effective (Table 1).

Silymarin was used as the reference positive control in these assays. This flavolignan mixture, isolated from the milk thistle *Silybum marianum*, has, indeed, demonstrated remarkable protective effects against human hepatic damage and against oxidative peroxidation in several experimental models [17,18]. It has also showed remarkable protective properties against CCl_4 induced hepatotoxicity [19,20]. It is, therefore, worth noting that the methanol extract of *F. chlamydicarpa* exhibited a preventive action against CCl_4-induced hepatotoxicity similar to that of silymarin (Table 1).

Antioxidant activity and/or free radical inhibition are important mechanisms for liver protection from CCl_4-induced damage [21-25]. The DPPH radical-scavenging activity test, the β-CLAMS and FRAP assays, as well as the inhibition of rat microsomal lipid peroxidation, are currently used by researchers for a rapid evaluation of the antiradical and antioxidant properties of a sample [26-29]. In an attempt to attribute the hepatoprotective properties of the methanol extract and isolated compounds **1**-**4** from *F. chlamydocarpa* to their antioxidant and radical scavenging effects, these samples were submitted to the indicated assays (Table 2). Trolox was used as the reference positive control.

Except for α-amyrin acetate (**1**), all the other three compounds, in particular luteolin (**4**), exhibited noticeable antioxidant and radical scavenging activities in the four tests.

In vitro cell cytotoxicity gives a very sensitive measure of the toxicity of plant extracts and their contents.

Table 2: Antioxidant and radical scavenging activities of isolated compounds **1-4** and the methanol extract of *F. chlamydocarpa*.[a]

	Biochemical antioxidant parameters (EC_{50} (µg/mL))			
Compounds and ME	**DPPH**	**IPL**	**β-CLAMS**	**FRAP**
1	NA	NA	NA	NA
2	6.0±3.8	11.9±2.7	8.9±3.0	8.0±2.9
3	5.7±1.6	10.9±3.0	NA	NA
4	5.0±2.0	7.7±1.6	6.9±2.9	5.1±1.8
ME	7.4±2.1	15.8±5.0	11.0±2.1	9.9±4.8
Trolox	3.5±1.7	5.6±3.6	4.9±2.0	4.1±1.7

[a]Values are EC_{50} ± SD of two experiments in triplicate. **DPPH**: radical scavenging activity; **IPL**: inhibition of lipid peroxidation; **FRAP**: ferric-reducing antioxidant power. **β-CLAMS**: inhibition of oxidative degradation of β-carotene. **ME**: methanol extract of *F. chlamydocarpa*. **Trolox**: reference antioxidant compound. NA: no activity shown at the dose tested. In each test, five sample concentrations were used: 12.5, 25, 50, 100, and 200 µg/mL.

Moreover, methods based on *in vitro* cell cultures have the advantage of relatively well-controlled variables and are generally accepted as very effective for biosafety testing. Moreover, compared with *in vivo* tests, their sensitivity is equal or even higher.

In the present study, we applied the MTT and LDH tests to evaluate the antihepatotoxic activity of the methanol extract of *F. chlamydocarpa* and isolated compounds **1-4** in rat hepatoma cell cultures. The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of cells [30]. In the lactate dehydrogenase (LDH) assay, leakage of the cytoplasmic located enzyme LDH into the extracellular medium is measured. In fact, the presence in the cell culture medium of the exclusively cytosolic enzyme LDH, is indicative of significant cell membrane damage [30].

Figures 1 and 2 show that until 100 µg/mL, cell viability increased by adding increasing amounts of either the methanol extract or isolated compounds **1-4**. Luteolin (**4**) was the most active sample and showed the lowest percentage of LDH leakage. Moreover, these results clearly show that α-amyrin acetate (**1**), although endowed with low antioxidant activity, displays a significant antihepatotoxic activity, as indicated by the ability to maintain a high cell viability during intoxication with CCl_4 and the significant inhibition of LDH leakage from intoxicated liver cells.

In conclusion, the results of this study demonstrate that the methanol extract of *F. chlamydocarpa* and isolated compounds **1-4** exhibit good hepatoprotective action towards CCl_4-induced hepatic damages in rats. Our data show that the effects of these samples may be due, at least in part, to their antioxidant activity, in addition to a combination of free radical scavenging ability and lipid peroxidation inhibitory properties. Further studies on other models and extensive clinical trials are needed to confirm these results *in vivo*

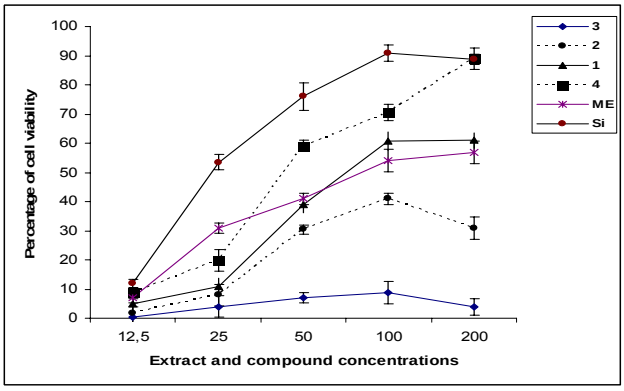

Figure 1: MTT assay in rat hepatoma cells after 24 h incubation with 12.5, 25, 50, 100, and 200 µg/mL of extracts and isolated compounds from *F. chlamydocarpa*. Reported values are the mean ± SD (standard deviations) of three independent experiments carried out in triplicate. **1-4**: isolated compounds from *F. chlamydocarpa*; **ME**: methanol extract; **Si**: sylimarin taken as an antihepatotoxic reference compound.

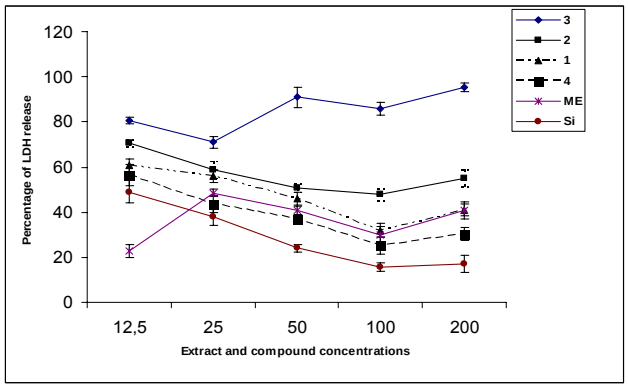

Figure 2: LDH leakage from rat hepatoma cells after 24 h incubation with 12.5, 25, 50, 100, and 200 µg/mL of extracts and isolated compounds from *F. chlamydocarpa*. LDH activity was measured in the supernatants. Reported values are the mean ± SD (standard deviations) of three independent experiments carried out in triplicate. **1-4**: isolated compounds from *F. chlamydocarpa*; **ME**: methanol extract; **Si**: sylimarin taken as an antihepatotoxic reference compound.

Experimental

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

Plant material: Samples of *F. chlamydocarpa* Mildbr. & Burr.stem bark were collected in Bahouan (west province of Cameroon) in July 2004. The plant was identified by Nana Victor of the Cameroon National Herbarium, where a voucher specimen is conserved under the reference number 35446/HNC.

Extraction and isolation of compounds: Sun-dried and powdered stem bark of *F. chlamydocarpa* (2 kg) was macerated in methanol (15 L) at room temperature for 48 h. The filtrate was concentrated under vacuum to give a dark green methanol extract (ME) (150 g). The ME (140 g) was subjected to flash column chromatography (FCC) on silica gel 60 and eluted with

a *n*-hexane-EtOAc gradient, followed by EtOAc-MeOH mixtures and MeOH to afford 6 fractions: fractions A (10 g, *n*-hexane), B (3 g, *n*-hexane-EtOAc, 3:1), C (1 g, *n*-hexane-EtOAc, 1:1), D (2 g, *n*-hexane-EtOAc, 1:3), E (5 g, EtOAc), F (50 g, EtOAc-MeOH, 9:1, followed by MeOH). Fraction A was subjected to FCC on silica gel 60. Elution with a *n*-hexane-EtOAc gradient yielded α-amyrin acetate [(**1**), 100 mg [31]. Fraction B and C were combined and subsequently separated by FCC, followed by preparative TLC to yield alpinumisoflavone [5-hydroxy-7-(*p*-hydroxyphenyl)-2,2-dimethyl-2*H*-6*H*-benzo[1,2-*b*:5,4-b']- dipyran-6-one (**2**) [32], genistein [4',5,7-trihydroxyflavone (**3**), 15 mg [33], and luteolin [3',4',5,7-tetrahydroxyflavone (**4**), 20 mg [34].

Animal experiments: Male Wistar albino rats from the Biochemistry Department (University of Yaounde I) animal house weighing 180-200 g were used for the assays. The study was performed with the approbation of the institutional ethical committee for animal care.

Rats were divided into 7 groups, each containing 5 rats: a) animals of group 1 (normal control group) were treated with a single dose of polyvinyl pyrrolidone (PVP 1% p.o) and maize oil (0.6 mL/kg, i.p) as CCl_4 vehicle; b) animals of group 2 (hepatotoxic group) were treated with a single dose of 1% PVP and then CCl_4 was administered (2 mL/ per i.p); c) animals of groups 3–6 were treated with the methanol extract of *F. chlamydocarpa* (25, 50, 100 and 200 mg/kg, p.o) suspended in PVP 1 h before intoxication with CCl_4, as described for group 2; d) animals of group 7 were treated with silymarin (100 mg/kg, p.o); a single dose of CCl_4 (2 ml/kg, i.p.) was administered 30 min after silymarin administration.

***Biochemical analyses*:** Twenty-four h after CCl_4 intoxication, animals were sacrificed by decapitation. Blood and liver samples were collected for evaluating the following biochemicals parameters: MDA by the Wilburg method [35], GSH by the Ellman method [36], serum GOT and serum GPT by using the standard Reitman-Frankel procedures [37].

In vitro *hepatoprotective activity using hepatoma cells*: The effects of isolated compounds and the methanol extract from *F. chlamydocarpa* on cell viability was assessed in a cell culture system using cells from rat Morris hepatoma cell line BS TCL 41. The antihepatotoxic effects were assayed on CCl_4-treated cells. The CCl_4 concentration used for cell culture treatment was previously determined and chosen for its capability to induce up to 75% cell culture mortality. The methanol extract and the isolated compounds from *F. chlamydocarpa* were not completely soluble in a hydrophilic medium like cell culture medium, so an emulsion in DMSO was prepared, which was then added to the cell culture medium. DMSO was 3% in cell culture medium. The cells were grown in Ham's F 10/F10 medium supplemented with 10% (v/v) inactivated fetal bovine serum, 2 mM L-glutamine and 1% penicillin/ streptomycin 100 x solution to prevent microbial contamination. Cells were maintained under an 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 in 96-well plates at a cell density of 35000 cells/well in serum-free culture medium. Twenty-four h after cell seeding, in separate experiments, a series of cells was simultaneously exposed to 12.5, 25, 50, 100 and 200 µg/mL of the methanol extract and isolated compounds, and 2.5 mM CCl_4 in fresh serum-free medium; a series of cells was treated with 2.5 mM CCl_4 in culture medium as positive controls and a series of cells was treated with culture medium-DMSO as negative controls.

MTT assay: Twenty-four h after cell seeding, cells were intoxicated with CCl_4 (2.5 mM) and incubated with 12.5, 25, 50, 100 and 200 µg/mL of methanol extract and isolated compounds for 24 h at 37°C. After removal of supernatants from each well, used to determine the Lactate Dehydrogenase leakage (LDH), 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 [38]. Subsequently, medium was removed, and cells were washed with phosphate-buffered saline solution and incubated for 15 min with acidic isopropanol to dissolve formazan crystals. The absorbance of the MTT formazan was read at 570 nm with a multiwell plate reader.

Lactate dehydrogenase assay: 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 instruction. In this test, the intensity of color obtained from the reaction is proportional to LDH activity. The absorbance was determined at 490 nm with a multiwell plate reader.

Radical-scavenging and antioxidant activities: The free radical-scavenging activities of the methanol extract and isolated compounds were evaluated by assessing the decrease in absorbance of DPPH (2,2-diphenyl-1-picrylhydrazyl) at 517 nm according to the Brand-Williams procedure [39]. The β-CLAMS (ß-Carotene-Linoleic Acid Model System) method was performed at 470 nm according to Miller [26]. The

FRAP (Ferric-Reducing Antioxidant Power) assay was performed at 595 nm according to Benzie & Strain [27]. Inhibition of lipid peroxidation (IPL) was investigated using rat liver microsomes isolated as described by Garle and Fry [40]. Non-enzymatic lipid peroxidation was initiated with ascorbate, as described by Nilsson [41], and assayed for thiobarbituric acid-reactive substances (TBARs) according to Wills [42]. In each assay, the methanol extract and the 4 isolated compounds from *F. chlamydocarpa* were tested at 12.5, 25, 50, 100, and 200 µg/mL; EC_{50} values, indicating the concentration of the sample required to scavenge 50% DPPH or to inhibit 50%, an oxidant mechanism, were estimated using Graph Pad Prism 3.0 software. Trolox was used as the reference antioxidant compound. Results are presented as means ± S.D. Total variation present in a set of data was analyzed through one-way analysis of variance (ANOVA) by using the Graph Pad Prism software; $P<0.05$ was taken as insignificant.

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