

In vitro and *In vivo* Hepatoprotective Study of *Inula crithmoides* L., *Pluchea dioscoridis* (L.) Desf. and *Phyllanthus reticulatus* Poir.

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Abstract

The hepatoprotective activities for methanolic extracts of *Inula crithmoides* L., *Pluchea dioscoridis* (L.) Desf. and *Phyllanthus reticulatus* Poir. were evaluated using *in vitro* and *in vivo* studies. The hepatoprotective activity was evaluated *in vivo* adopting a carbon tetrachloride induced liver toxicity model in adult male albino rats, using silymarin as a positive control. The study included biochemical measurement of ALT and AST in serum samples and histopathological examination. The hepatoprotective compounds were isolated from the biologically active fractions using different chromatographic techniques. The isolated compounds were identified using different spectroscopic techniques including UV, IR, MS, ¹H-NMR and ¹³C-NMR. *Inula crithmoides* L. possessed the highest hepatoprotective activity among the tested plants. Ethyl acetate fraction of *Inula crithmoides* L. exhibited the highest activity followed by *n*-butanol, methylene chloride, and petroleum ether fractions. Quercetin, 3,5-Dicaffeoylquinic acid, chlorogenic acid, α -amyrin, β -sitosterol and β -sitosterol-3-*O*- β -D-glucopyranoside were isolated and identified. 3,5-Dicaffeoylquinic acid and chlorogenic acid were isolated for the first time from *Inula crithmoides* L. aerial parts. The hepatoprotective activity of *Inula crithmoides* L. could be attributed to its content of dicaffeoylquinic acids and their derivatives as chlorogenic acid.

Keywords: Chlorogenic acid, 3,5-Dicaffeoylquinic acid, Hepatoprotective, Histopathology, *Inula crithmoides* L., MTT assay.

1. INTRODUCTION

Liver diseases remain one of the major threats to public health and are a worldwide problem [1]. They are mainly caused by some drugs like acetaminophen (in large doses), excess consumption of alcohol, infections and autoimmune disorders [2]. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages [2]. Liver injuries induced by carbon tetrachloride are the best characterized mechanisms of xenobiotic-induced hepatotoxicity and such models are commonly used to screen the antihepatotoxic and hepatoprotective activity of various drugs [3].

Natural products produced by medicinal plants and their extracts play a key role in human health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant material [4]. Scientific studies available on medicinal plants indicate that promising phytochemicals can be developed for many health problems [5]. The present study was carried out to study the potential of some Egyptian plants as hepatoprotective agents.

2. MATERIAL AND METHODS

2.1. Plant materials

Aerial parts of *Inula crithmoides* L., *Pluchea dioscoridis* (L.) Desf. were collected from Egypt International Road, Balteem area, while that of *Phyllanthus reticulatus* Poir. were collected from experimental farm of Faculty of Agriculture, Menofia University in March 2008. They were identified by Prof. Metwally Mazroaa, Faculty of Agriculture, Menofia University, Egypt. Voucher specimens were deposited in the Herbarium of Faculty of Pharmacy, Pharmacognosy Department, Tanta University.

The three plants were separately dried in oven at 40°C, reduced to a fine powder and stored in tightly closed containers.

2.2. Experimental animals

Adult male albino rats (150-180 g) were used. The study guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals was conducted.

2.3. Materials for biological study

Assay kits for analysis of biochemical parameters were purchased from Biodiagnostic, Egypt. Silymarin was purchased from Sigma Pharmaceutical Industry Company, Egypt.

2.4. Chromatographic materials and Chemicals

Precoated TLC silica gel plates G 254 F (E. Merck, Germany), silica gel for column chromatography (E. Merck, 70-230 mesh), sephadex LH-20 (Sigma-Aldrich chemical Co., USA), reversed phase octadecylsilyl-silica gel (RP-C18, Merck, Germany) and diaion HP-20 (Mitsubishi Chemical, Japan) were used. All chemicals used were of high analytical grade.

2.5. Apparatus

EI/MS spectra were recorded on Thermo Scientific ISQ Singl Quadrupole MS, USA. ¹H- and ¹³C-NMR were recorded on a Bruker Avance III 400 MHz Spectrometer, Germany. Chemical shifts were given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. IR spectra were recorded with a Jasco FT/IR-6100 spectrophotometer. Jasco UV/Vis spectrophotometer, V-530 (Japan) was used for measurement of ALT and AST in

serum samples. Melting point determination was carried out using Gallenkamp melting point apparatus.

2.6. General experimental procedures

2.6.1 Preparation of plant extracts

Methanolic extracts of: *Inula crithmoides* (L.), *Pluchea dioscoridis*(L.) Desf., and *Phyllanthus reticulates* Poir., were separately dissolved in 0.5% aqueous carboxymethyl cellulose.

- *In vivo* hepatoprotective activity

Two parameters were used for investigation of the hepatoprotective effect of the plants:

i- Determination of serum ALT and AST

Ninety six (96) male albino rats were divided into 12 groups each of 8 rats. Group 1 was received 5 ml/kg b.w. of 0.5 % CMC in water (normal control), group 2 (hepatotoxic) was received 2 ml/kg b.w. of 25% CCl₄ in corn oil, group 3 (positive control) was received 100 mg/kg of silymarin, suspended in 5 ml of 0.5 % aqueous CMC once daily for 3 days followed by 2 ml/kg b.w. of 25% CCl₄ in corn oil on the fourth day. The other groups 4 to 12 were separately treated with 100, 200 and 300 mg/kg in 0.5 % aqueous CMC of the plants extracts for 3 days followed by 2 ml/kg b.w. of 25 % CCl₄ in corn oil on the fourth day. The doses of extracts were chosen based on published biological studies.^[6-8] Blood was collected via cardiac puncture. Alanine transaminase (ALT) and aspartate transaminase (AST) levels were determined in the serum. The assay was performed according to the method described by Reitman and Frankel.^[6] using Jasco UV/Vis spectrophotometer, V-530 (Japan).

Comparison between different groups was carried out by analysis of variance test (ANOVA) followed by Tukey test. The level of significance was set at $P \leq 0.05$. The Standard Package of Social Sciences (SPSS) computer software was used to carry out the statistical analysis.

ii-Histopathological analysis

It was carried out on liver sections, of killed rats after blood collection, and stained with hematoxylin and eosin stain (H & E) to be studied by light microscope [7].

- *In vitro* hepatoprotective study of *Inula crithmoides* L. methanolic extract and its different fractions

A modification of the technique described by Seglen [8]. was used for the hepatocyte isolation. The cytotoxic activity of the methanolic extract and its fractions was attempted by using Hansen et al [9]. procedures for determination of mitochondrial synthesis by micro culture tetrazolium (MTT) assay. The method described by Hansen et al [9]. was based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell which make cleavage of MTT to a blue formazan derivative. The absorbance was measured using microplate reader at 540 nm.

- *In vitro* hepatoprotective activity against CCl₄ induced toxicity

The above procedure was adopted using equal aliquots of the methanolic extract or its fractions and CCl₄. The study was carried out using the method described by Vijayan et al [10].

2.6.2. Isolation of pure compounds

Methylene chloride fraction of *Inula crithmoides* L. aerial parts (7 g) was chromatographed on a silica gel column (3.5 × 46.5 cm, 210 g) using gradient elution method, starting with methylene chloride and increasing polarity by adding increasing amounts of methanol. Fractions (100 ml each) were monitored by TLC silica gel sheets using solvent system: CH₂Cl₂: MeOH, 99:1 (v/v). Fractions eluted with 100% CH₂Cl₂ (48 mg) were combined, evaporated, and crystallized from methylene chloride/ether mixture to yield Compound **1** (24 mg). Compound **1** was obtained as a white powder (yield 24 mg), m.p. (186-188 °C), TLC using silica gel G 254 F: R_f= 0.45 in solvent system: CH₂Cl₂: MeOH, 99:1 (v/v), EI-MS: m/z (% rel. int.): 426 (5) [M]⁺, 218 (68), 203 (24), 189 (22), 147 (24), 133 (35), 119 (53), 107 (62), 95 (73), 81 (77), 69 (89), 55 (100).

Fractions eluted with 100% CH₂Cl₂ were combined, evaporated (120 mg), and subjected to preparative TLC using CH₂Cl₂: MeOH (97:3). The bands were visualized by the aid of a guide spot sprayed with 10 % sulphuric acid, scrapped off and eluted with CH₂Cl₂ to yield 9 mg of compound **2**. Compound **2** was obtained as white needle crystals by crystallization several times from methylene chloride: petroleum ether mixture, m.p. (137-139 °C), TLC using silica gel G 254 F: R_f= 0.40 in solvent system: CH₂Cl₂: MeOH, 97:3 (v/v). EI-MS: m/z (% rel. int.): 414 (25) [M]⁺, 396 (10), 381 (4), 329 (12), 303 (9), 273 (10), 255 (14), 213 (15), 159 (30), 145 (41), 105 (61), 81 (80), 55 (100). IR (KBr)^{cm-1}: 3430 (O-H stretching), 2932 and 2860 (C-H stretching), 1633 (C=C stretching), 1460 (CH₂-C bending), 1378 (CH₃-C bending), 962 (C-O stretching) and 877 (C-H bending).

Fractions eluted with 96% CH₂Cl₂ in MeOH (243 mg) were combined, evaporated, and crystallized to yield compound **3** (11 mg). Compound **3** was obtained after repeated crystallization from methanol/ acetone mixture. The compound was obtained as a white amorphous powder, m.p. (275-277°C), TLC using silica gel G 254 F: R_f= 0.33 in solvent system: CH₂Cl₂- MeOH, 90:10 (v/v).

Ethyl acetate fraction of aerial parts of *I. crithmoides* L. (3 g) was chromatographed on a silica gel column (2.5 × 60 cm, 145 g) by gradient elution method, starting with methylene chloride and the polarity was increased using methanol. Fractions eluted with 91% CH₂Cl₂ in MeOH (120 mg) were combined, evaporated, and subjected to preparative TLC using toluene:ethylacetate:formic acid 30:70:5 for elution. The major band was visualized by UV light, scrapped off, and eluted with methanol to give compound **4** (11 mg). Compound **4** was obtained as a yellow powder, m.p. (316-318°C), TLC using silica gel G 254 F: R_f= 0.72 in solvent system: toluene:ethyl acetate:formic acid, 30:70:5 (v/v). UV-Visible λ_{max} (nm) MeOH: 256, 371; MeOH+NaOH: 290, 426; MeOH+AlCl₃: 271, 455; MeOH+AlCl₃+HCl: 266, 302 (sh.), 427, 361 (sh.); MeOH+NaOAc: 271, 325 (sh.), 381; MeOH+NaOAc/Boric acid: 261, 389. EI-MS: m/z (% rel. int.): 302 (58) [M]⁺, 286 (9), 274 (13), 257 (5), 245 (3), 229 (7), 217 (3), 152 (8), 150 (5), 137 (16), 83 (50), 73 (35), 57 (100).

Column fractions eluted with 88-80% methylene chloride in MeOH were pooled and evaporated under vacuum. The residue (2 g) was rechromatographed on silica gel column (1.7 × 40 cm, 65 g) using gradient elution of CH₂Cl₂:MeOH. The pooled fractions eluted with methylene chloride in methanol (85–70%) were evaporated (320 mg) then chromatographed on a reversed phase C18 silica gel column (1.6 × 22 cm, 17 g). The elution was carried out using 10% methanol in water and increasing methanol concentration. Fractions eluted with 15% methanol in water were pooled to give compound **5** (15 mg) which was crystallized from MeOH /H₂O mixture (7:3). Compound **5** was obtained as a yellowish white powder, m.p. (170-172 °C). TLC using silica gel G 254 F: R_f= 0.75 in solvent system: *n*-butanol:acetic acid:water, 40:10:50 (v/v) upper layer. UV-Visible λ_{max} (nm) MeOH: 328, 298 (sh.), 244 (sh.), 219 nm; MeOH+AlCl₃: 359, 309 (sh.), 261, 227; MeOH+AlCl₃+HCl: 330, 298 (sh.), 244 (sh.), 218 nm. ¹H NMR (400 MHz, CD₃OD) and ¹³C NMR (100 MHz, CD₃OD) data were determined and presented in Table (3). *n*-butanol fraction of aerial parts of *I. crithmoides* L. (16.5 g) was suspended in deionized water and applied to diaion HP-20 column (2.5 × 45 cm, 85 g). The column was eluted with deionized water followed by H₂O-MeOH (50:50), then (100%) methanol and finally washed with acetone. The fractions eluted with 50% methanol were evaporated and freeze dried to give a brown residue (2.5 g). The dried residue was chromatographed on a silica gel column (3.2 × 36.5 cm, 100 g) using gradient elution, starting with methylene chloride and increasing the polarity with methanol. Column fractions eluted with 85-75 % methylene chloride in MeOH were collected, concentrated and the residue (188 mg) was subjected to reversed phase C18 silica gel CC (1.6 × 22 cm, 17 g) starting elution with 10% methanol in water then increasing concentration of methanol. Fractions eluted with 10% (55 mg), were combined, evaporated and subjected to sephadex LH-20 column (1.5 × 39 cm, 20 g). Elution was carried out using

methanol:water (95:5). Fractions (14-19), 1 ml each, were combined and evaporated to give a residue (21 mg), which was crystallized to yield compound **6** (13 mg). Compound **6** was obtained as a yellowish white powder, m.p. (208-211°C), TLC using silica gel G 254 F, R_f=0.39 in solvent system: *n*-butanol:acetic acid:water, 40:10:50 (v/v) upper layer. UV-Visible λ_{max} (nm) MeOH: 329, 300(sh.), 243(sh.), 217 nm; MeOH+AlCl₃: 359, 311(sh.) nm, 262, 226; MeOH+AlCl₃+HCl: 329, 297 (sh.), 244 (sh.), 218 nm. ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD) data were determined and presented in Table (4).

3. RESULTS AND DISCUSSION:

3.1. In vivo study of the hepatoprotective activity

Aerobic organs such as the liver employ a battery of defense mechanisms, such as antioxidant enzymes to prevent oxidative tissue damage. Thus, the protective agents against CCl₄-induced liver injury exerts their actions by the impairment of CCl₄ mediated lipid peroxidation through the inhibited generation of free radical derivative [11] or due to the antioxidant activity of the protective agent itself [12].

Intraperitoneal injection of 25% carbon tetrachloride at a dose of 2 ml/kg b.w. significantly increase the serum activities of ALT and AST. It was reported that the most accepted hypothesis of hepatotoxicity for CCl₄ was the bioactivation of the CCl₄ molecules to the trichloromethyl toxic free radical by certain isoenzymes of cytochrome 450 [13].

Carboxymethyl cellulose suspension of *Inula crithmoides* L., *Pluchea dioscoridis* (L.) Desf., and *Phyllanthus reticulatus* Poir. methanolic extracts of aerial parts at different dose levels significantly decreased the ALT and AST serum levels, compared to the normal and hepatotoxic animals (Table 1).

Table 1: Effect of different doses of methanolic extracts of *Inula crithmoides* L., *Pluchea dioscoridis* (L.) Desf. and *Phyllanthus reticulatus* Poir. aerial parts on ALT and AST serum levels in rats with CCl₄ induced hepatotoxicity

Group/ Dose in mg/kg (b.w.)	*ALT serum level units/ml	*AST serum level units/ml
Normal (-ve control)	50.96 ± 2.7	68.43 ± 3.66
Hepatotoxic (CCl ₄ group)	382.12 ± 19.2 a	444.12 ± 21.66 a
Silymarin (+ve control) 100 mg	71.79 ± 6.73 b	88.98 ± 7.57 b
<i>I. crithmoides</i> L.		
100 mg	115.1 ± 11.55 a b c	142.28 ± 11.98 a b c
200 mg	105.13 ± 10.16 a b c	129.36 ± 9.05 a b c
300 mg	87.13 ± 11.12 a b	112.92 ± 8.09 a b
<i>P. dioscoridis</i> (L.) Desf.		
100 mg	275.37 ± 8.99 a b c	330.82 ± 18.1 a b c
200 mg	254.1 ± 8.7 a b c	315.14 ± 17.23 a b c
300 mg	233.55 ± 11.37 a b c	289.28 ± 14.97 a b c
<i>P. reticulatus</i> Poir.		
100 mg	194.42 ± 11.3 a b c	235.34 ± 12.84 a b c
200 mg	179.98 ± 7.87 a b c	210.2 ± 15.53 a b c
300 mg	138.47 ± 10.5 a b c	169.05 ± 11.7 a b c

*Mean ± S.D

a Significant difference from control group (p<0.05)

b Significant difference from hepatotoxic group (p<0.05)

c Significant difference from silymarin (+ve control) group (p<0.05)

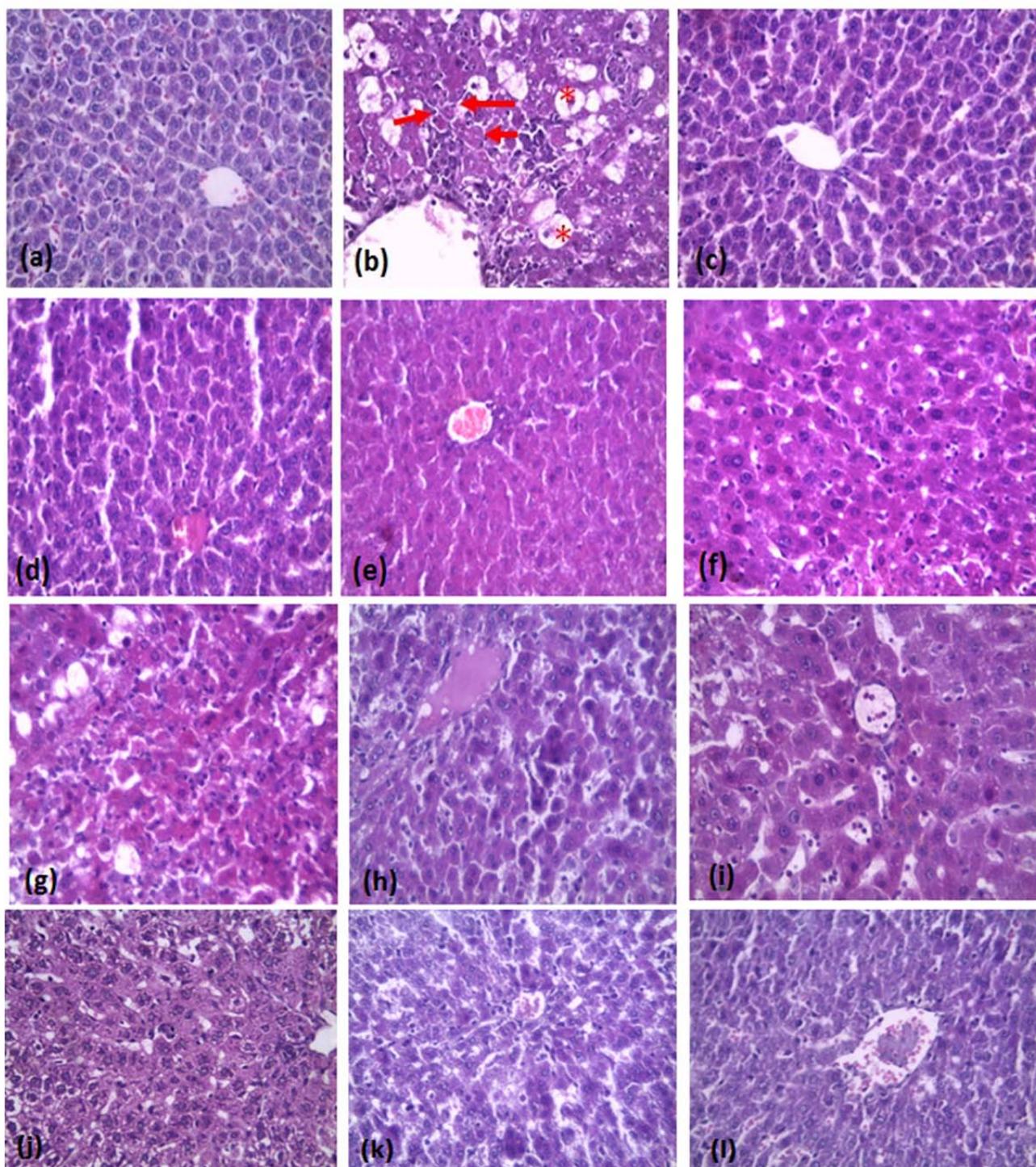


Figure 1: Light micrographs of hematoxylin and eosin staining $\times 400$. (a) Normal control (b) CCl₄-induced liver toxicity showing extensive vacuolar degeneration and apoptotic figures (asterisks) and necrotic cells with mononuclear cellular infiltration (arrows). (c) Silymarin + CCl₄ showing apparently normal hepatic architecture. (d) *Inula crithmoides* L. 100 mg + CCl₄ showing dilatation of sinusoids and central vein (e) *Inula crithmoides* L. 200 mg + CCl₄ and (f) *Inula crithmoides* L. 300 mg + CCl₄ showing apparently normal hepatocytes and hypertrophied kupffer cells. (g) *Pluchea dioscoridis* (L.) Desf. 100 mg + CCl₄ showing necrotic cells (highly eosinophilic cells), karyolytic cells and mononuclear cellular infiltration. (h) *Pluchea dioscoridis* (L.) Desf. 200 mg + CCl₄ showing scattered focal necrotic cells, hypertrophied kupffer cells and dilated blood sinusoids. (i) *Pluchea dioscoridis* (L.) Desf. 300 mg + CCl₄ showing swollen cells (cloudy swollen), mild vacuolar damage, hypertrophied kupffer cells and dilated blood sinusoids. (j) *Phyllanthus reticulatus* Poir. 100 mg + CCl₄ showing vacuolar degeneration, dilated engorged central vein and blood sinusoids with hypertrophied kupffer cells and focal mononuclear cellular infiltration. (k) *Phyllanthus reticulatus* Poir. 200 mg + CCl₄ showing focal central zonal necrosis, dilated central veins and blood sinusoids and hypertrophied kupffer cells. (l) *Phyllanthus reticulatus* Poir. 300 mg + CCl₄ showing mild to moderate dilatation of central veins and blood sinusoids with mild vacuolar degeneration of hepatocytes.

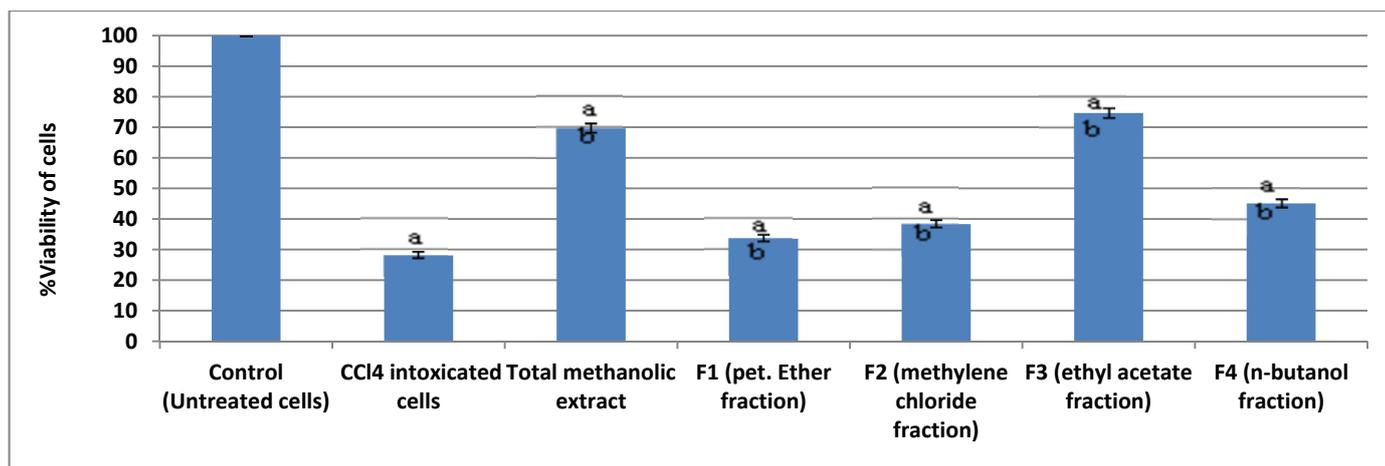


Figure 2: Effects of total methanolic extract and different fractions from *Inula crithmoides* L. on hepatocytes viability.

The different doses of *Inula crithmoides* L. showed significant effect compared with the doses of the other tested plants. The maximum inhibition of hepatotoxicity was detected in the highest dose of *Inula crithmoides* L. (300 mg) which was comparable to silymarin as shown in Table (1).

The results of histopathological study illustrated in photomicrographs (Figure1) confirmed the biochemical parameters and presented a good correlation between the ALT and AST serum levels and the histopathological findings. The results showed that methanolic extract of *Inula crithmoides* L. had the highest protective effect against hepatotoxicity. Methanolic extracts of *Phyllanthus reticulatus* Poir. and *Pluchea discoridis* (L.) Desf. presented lower hepatoprotective activities. The results of this study also showed that the hepatoprotective effect was dose dependent. Bioassay guided fractionation was carried on *Inula crithmoides* L. aerial parts as a prelude to isolate the active compounds from its fractions.

3.2. In vitro study of the hepatoprotective activity

The highest percentage viability was observed in ethyl acetate fraction (74.65 %), followed by total methanolic extract (69.67 %), *n*-butanol fraction (45.11 %), methylene chloride fraction (38.34 %) and at last petroleum ether fraction (33.54 %) as shown in Table (2) and illustrated in Figure (2).

Table 2: Protective effect of *Inula crithmoides* L. methanolic extract and different fractions on CCl₄ induced toxicity in rat hepatocytes.

Treatment	% Viability of cells*
Control (untreated cells)	100
CCl ₄	28.11 ± 1.02 a
Total methanolic extract	69.67 ± 1.46 a b
Petroleum ether	33.54 ± 1.05 a b
Methylene chloride	38.34 ± 1.21 a b
Ethyl acetate	74.65 ± 1.55 a b
<i>n</i> -Butanol	45.11 ± 1.32 a b

*Average of six independent determinations, values are expressed as mean ± S.E.M. a Significant difference from control group (untreated cells) (P < 0.05)

b Significant difference from CCl₄ intoxicated cells (P < 0.05)

3.3. Phytochemical investigation

Further phytochemical investigation was performed on the most active fractions, to isolate and identify the active compounds. This study resulted in the isolation of six compounds. Their structures were established using physical and spectroscopic techniques including: melting points, UV, IR, ¹H-NMR, ¹³C-NMR and MS. Compound **1** was obtained as a white powder (yield 24 mg), m.p. (186-188 °C). By comparison the EI-MS and ¹H-NMR (400 MHz, CDCl₃) and ¹³C-NMR (100 MHz, CDCl₃) data with those reported in the literature, the compound was identified as 3β-hydroxy-urs-12-en-3-ol [14-16].

Compound **2** was obtained as white needle crystals (yield 9 mg), m.p. (137-139 °C). By comparison EI-MS, IR, and ¹H-NMR (400 MHz, CDCl₃) data with that reported in the literature, the compound was identified as 3-β-stigmast-5-en-3-ol (β-sitosterol) [17-18].

Compound **3** was obtained as a white amorphous powder, (yield 11 mg), m.p. (275–277 °C). TLC using silica gel G 254 F:R_f= 0.33 in solvent system:CH₂Cl₂-MeOH, 90:10 (v/v). By comparison ¹H-NMR (400 MHz, DMSO-*d*₆) data with respective values reported in the literatures, compound **3** was identified as β-sitosterol-3-*O*-β-D-glucopyranoside, also known as daucosterol [19-20].

Compound **4** was obtained as a yellow powder (yield 11 mg), m.p. (316-318°C). By comparison of the UV shift reagent values with those reported in literature [21-22] and mass spectral data [23-24], compound **4** was identified as quercetin.

Compound **5** was obtained as a yellowish white powder (yield 15 mg), m.p. (170-172 °C), TLC using silica gel G 254 F:R_f= 0.75 in solvent system:*n*-butanol:acetic acid:water,40:10:50 (v/v) upper layer.UV-Visible λ_{max} (nm) MeOH: 328, 298(sh.), 244(sh.),219 nm; MeOH+ AlCl₃: 359, 309(sh.), 261, 227; MeOH+AlCl₃+HCl: 330, 298(sh.), 244(sh.), 218 nm. The UV spectra in methanol before and after the addition of AlCl₃ and AlCl₃/HCl provided a preliminary evidence for compound **5** to be a caffeic acid derivative with *O*-dihydroxyl group [25].

$^1\text{H-NMR}$ (400 MHz, CD_3OD) and $^{13}\text{C-NMR}$ (100 MHz, CD_3OD) data (Table 3) suggested that compound 5 could be identified as 3,5-Dicaffeoylquinic acid. This was confirmed by co-TLC with an authentic sample and comparing these data to those published in literature [26-28]. This compound was isolated for the first time from *Inula crithmoides* L.

Table 3: $^1\text{H-NMR}$ (CD₃OD, 400 MHz) and $^{13}\text{C-NMR}$ (CD₃OD, 100 MHz) spectral data of compound 5

Units, atoms	δ H ppm (<i>J</i> in Hz)	δ 13C (ppm)
Quinic acid moiety		
1	1.97 (2H, <i>m</i>)	74.7
2	4.07 (1H, <i>m</i>)	36.8
3	3.62 (1H, <i>dd</i> , <i>J</i> =3.1, 8.5)	69.9
4	5.23 (1H, <i>ddd</i> , <i>J</i> =4.4, 8.8, 9.0)	70.6
5		72.1
6	2.12 (2H, <i>m</i>)	37.4
7		175.6
Caffeoyl moiety		
1'		126.4
2'	6.94 (1H, <i>d</i> , <i>J</i> = 1.9)	113.8
3'		145.4
4'		148.2
5'	6.67 (1H, <i>d</i> , <i>J</i> =8.2)	115.1
6'	6.85 (1H, <i>dd</i> , <i>J</i> =1.9, 8.2)	121.6
7'	7.45 (1H, <i>d</i> , <i>J</i> = 15.9)	145.7
8'	6.16 (1H, <i>d</i> , <i>J</i> =15.9)	113.9
9'		167.3

Table 4: $^1\text{H-NMR}$ (CD₃OD, 400 MHz) and $^{13}\text{C-NMR}$ (CD₃OD, 100 MHz) spectral data of compound 6

Units, atoms	δ H ppm (<i>J</i> in Hz)	δ 13C (ppm)
Quinic acid moiety		
1		73.4
2	2.08 (2H, <i>m</i>)	34.7
3	5.32 (1H, <i>m</i>)	71.3
4	3.87 (1H, <i>d</i> , <i>J</i> =7.5)	69.4
5	5.30 (1H, <i>m</i>)	70.7
6	2.22 (2H, <i>m</i>)	36.5
7		176.3
Caffeoyl moiety		
1'		126.6
2'	6.96 (2H, <i>s</i>)	114.2
3'		145.9
4'		148.1
5'	6.68 (2H, <i>d</i> , <i>J</i> =8.2)	115.1
6'	6.86 (2H, <i>m</i>)	121.7
7'	7.50 (2H, <i>d</i> , <i>J</i> =15.9)	145.4
8'	6.20 (2H, <i>d</i> , <i>J</i> =15.9)	113.8
9'		167.3
1''		126.4
2''	6.96 (2H, <i>s</i>)	113.9
3''		145.9
4''		148.2
5''	6.68 (2H, <i>d</i> , <i>J</i> =8.2)	115.1
6''	6.86 (2H, <i>m</i>)	121.6
7''	7.50 (2H, <i>d</i> , <i>J</i> =15.9)	145.6
8''	6.20 (2H, <i>d</i> , <i>J</i> =15.9)	113.8
9''		167.0

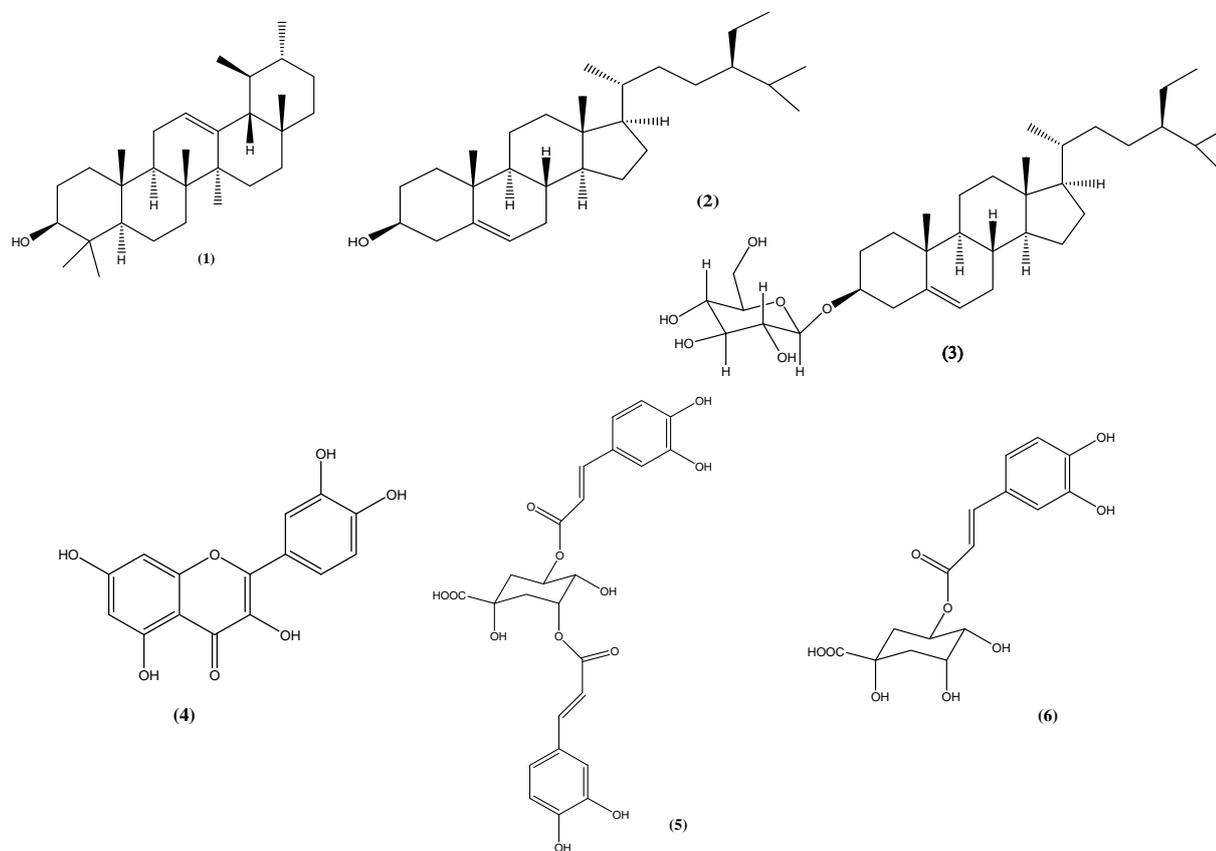


Figure 3: Chemical structures of the compounds isolated from the biologically active fractions.

Compound **6** was obtained as a yellowish white powder (yield 13 mg), m.p. (208-211°C), TLC using silica gel G 254 F: $R_f = 0.39$ in solvent system: *n*-butanol:acetic acid:water, 40:10:50 (v/v) upper layer. UV-Visible λ_{max} (nm) MeOH: 329, 300(sh.), 243(sh.), 217 nm; MeOH+AlCl₃: 359, 311(sh.) nm, 262, 226; MeOH+AlCl₃+HCl: 329, 297 (sh.), 244 (sh.), 218 nm. Notable features of the UV spectra included a prominent peak at 329 nm and a shoulder at 300 nm, characteristic of the phenylpropanoids [29]. Shift with AlCl₃ (30 nm) and AlCl₃/ HCl also suggested the presence of *O*-dihydroxyl system.

By comparison of m.p., co-TLC, UV, superimposable IR with authentic sample, ¹H-NMR and ¹³C-NMR data (Table 4) with the published data, compound **6** was identified as 5-Caffeoylquinic acid (chlorogenic acid) [30-31]. This is the first report for the isolation of chlorogenic acid from *Inula crithmoides* L.

Some studies have shown potential beneficial properties of chlorogenic acid to humans such as antioxidant, and hepatoprotective activities [32]. Dicafeoylquinic acids derivatives as chlorogenic acid are responsible for hepatoprotective potential of some herbal extracts and pure isolates [33-34].

4. CONCLUSION:

In vitro and *in vivo* hepatoprotective studies of the methanolic extracts of three Egyptian plants namely, *Inula crithmoides* L. (*Asteraceae*), *Pluchea dioscoridis* (L.) Desf. (*Asteraceae*), and *Phyllanthus reticulatus* Poir. (*Euphorbiaceae*) were carried out. The results revealed that *Inula crithmoides* L. possessed the highest hepatoprotective activity among the tested plants. Bioassay-guided fractionation was carried out and the results revealed that the hepatoprotective activity of *Inula crithmoides* L. could be attributed to its content of dicafeoylquinic acids and their derivatives as chlorogenic acid. Medicinal plants can represent a cheap source for preparing valuable pharmaceutical drugs that are becoming of global importance as hepatoprotective drugs.

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