Nermeen F. Farag et al /J. Pharm. Sci. & Res. Vol. 7(9), 2015, 633-639

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Protective Effect of *Chrysanthemum Pacificum Against* CCl_4-Induced Injury on the Human Hepatoma Cell Line (Huh7)

Nermeen F. Farag, Enas H. Abdelrahman*, Shadia M. Azzam and El-Sayeda A. El-Kashoury

Pharmacognosy Department,
Faculty of Pharmacy, Cairo University,
Kasr El-Aini Street, P.B. 11562, Cairo, Egypt

Abstract

Aims:
Evaluation of hepatoprotective, free radical scavenging potentials of the plant parts and isolation, characterization of major active compounds.

Methods:
An in-vitro assay using human hepatoma cell line (Huh7) was used to evaluate the effect of methanol extract of *C.pacificum* Nakai parts (flowers, aerial parts, roots) and fractions of flowers (10,100,1000 µg/ml) on enzymes activities before and after exposure of the cells to carbon tetrachloride (CCl_4).

Results:
Among the tested parts, flowers ethanol extract (100 µg/ml) exhibited the highest significant increase in superoxide dismutase (SOD) activity (237.18±6.5U/ml) and liver glutathione (GSH) level (25.42±1.74 mg/dl). Furthermore it showed the highest free radical scavenging activity (IC_{50}, 1.2 mg/ml) even more potent than silymarin (IC_{50}, 1.84 mg/ml). Ethyl acetate (EtOAc) and butanol (n-BuOH) fractions of flowers (100 µg/ml) exhibited higher potency than other fractions and silymarin in normalizing alanine transaminase (ALT) (11.42±0.43 & 13.68±0.87 U/ml respectively) and SOD (276.7±1.31&276.7±2.19 U/ml respectively) activities. Moreover, butanol fraction was more effective in neutralizing GSH level (18.4 ±0.10 mg/dl) compared to silymarin (16.73±1.33 mg/dl). Bioassay guided fractionation led to the isolation of luteolin, luteolin-7-O-glucuronide, luteolin-7-O-β-rutinoside (scolymoside) and 1,5-di-O-caffeoylquinic acid (cynarin), the latter compounds are active hepatoprotectives in artichoke.

Conclusion:
This is the first report for the protective and radical scavenging potentials of *C. pacificum* which may partly attributed to the presence of active phenolics as cynarin.

Keywords: Antioxidant, *Chrysanthemum pacificum*, hepatoprotective, Huh7

INTRODUCTION

Oxidative stress is considered a main mechanism in contributing to the initiation and progression of hepatic damage in a variety of liver disorders (1-3). Many plant phenolics have potent antioxidant and hepatoprotective effects (4-7). Genus *Chrysanthemum* has been reported to contain significant amounts of flavonoids and hydroxycinnamoyl-quinic acids which demonstrate radical-scavenging and anti-hepatotoxic activities(5-7,8-12). Our previous study on *C. pacificum* Nakai family Asteraceae(13) ascertained the metabolites profiling in the plant on an organ basis using UPLC-MS. Hydroxycinnamic acid derivatives and flavonoids were detected as the most abundant phytoconstituents. Herein we report on for the first time an *in-vitro* assay using the human hepatoma cell line (Huh7) to evaluate the possible hepatoprotective, antioxidant potentials of *C. pacificum* organs against CCl_4-induced injury. Furthermore, this study correlates the activity among organs with their phenolics and flavonoids content.

MATERIALS AND METHODS

General techniques

The antioxidant activity was determined using Jenway double beam spectrometer (UK). Shimadzu UV-1650 PC was used for recording UV spectra and Chrom Tech model (CT-2400) for determination of total phenolic and flavonoid content. NMR analyses were run on Varian VNMRS 600 NMR spectrometer (Bruker Daltonics, Bremen, Germany) ^{1}H-NMR, 600 MHz, ^{13}C-NMR, 125 MHz**.** or Bruker NMR-spectrophotometer **(**Microanalytical Unit, Faculty of Pharmacy, Cairo university), ^{1}H-NMR, 400 MHz, ^{13}C-NMR, 100 MHz, Japan relative to TMS in CD_3OD or DMSO. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), Silica gel 60 H for VLC (E-Merck, Darmstadt, Germany), silica gel 60 for column chromatography (Fluka,70–230 mesh, Germany)and silica gel 100 C_{18}–reversed phase (70–230 mesh, Fluka) were used. Pre-coated silica gel 60 F_{254} plates (Merck, Germany) were used for TLC.

Plant material

Samples of the flowers capitulum, aerial parts and roots were collected during 2012-2013 from the plants cultivated in the Experimental Station of Medicinal plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University. The plant was kindly identified by Mrs. Therease Labib, taxonomy specialist, Orman Botanical Garden, Giza, Egypt. A voucher specimen (27.2.2014) was deposited at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Chemicals and standards

Silymarin, carbon tetrachloride (CCl_4) and dimethyl sulfoxide were purchased from Sigma–Aldrich chemicals Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin 0.25%, penicillin G, streptomycin and phosphate-buffered saline were obtained from Gibco Invitrogen (Carlsbad, CA, USA). Kits for measurement of AST, ALT, SOD activities and GSH level were purchased from Biodiagnostic for diagnostic reagents (Dokki, Giza, Egypt).

2, 2-Diphenyl-1-picrylhydrazyl (DPPH reagent), trolox and gallic acid (Sigma-Aldrich, St. Louis, MO, USA) for free radical scavenging estimation. Folin-Ciocalteu reagent used for determination of total polyphenolic content obtained from Loba-Chemie (Mumbai, india).

Cell culture

Human hepatoma cancer cells (Huh-7) obtained from VACSERA (Dokki, Giza, Egypt). Cells were maintained in DMEM medium supplemented with 100 mg/ml streptomycin, 100units/ml penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% CO_2 atmosphere at 37 C.

Hepatoprotective potential estimation

The dried powders of flowers, aerial parts and roots were macerated in 70% ethanol till exhaustion and dried under vacuum**.** The fresh flowers of *C. pacificum* Nakai was extracted with 70% ethanol at room temperature till exhaustion, the extract, in each case, was dried under vacuum till dryness. Dry ethanol extract was re-suspended in H_2O and successively extracted with PE, $CHCl_3$, EtOAc and *n*-BuOH saturated with water till exhaustion. Each extract was concentrated under reduced pressure till dryness. The extracts of the different organs and fractions of flowers extract were used for the hepatoprotective assay. To assess the hepatoprotective effect of the tested samples, exponentially growing cells were collected using 0.25% trypsin-EDTA and plated in 6-well plates at 10^5cells/well. Cells were treated with various concentrations of the plant extracts (10, 100, 1000 µg/ml in phosphate-buffered saline) for one hour, followed by the addition of 40 mM CCl_4 in 0.05% dimethyl sulfoxide and subsequent incubation for two hours. The supernatant was collected and assayed for AST, ALT, SOD activities and GSH level. Silymarin was used as a reference agent. All variables were measured in triplicates and the experiment was repeated three times. Results were expressed as mean ± standard deviation (S.D.), calculated from n = 3. Data were analyzed using one-way analysis of variance (ANOVA), followed by Turkey-Kramer's post hoc test. Statistical significance was accepted at a level of $p<0.001$.

Free radical scavenging activity estimation

Lyophilised 70% methanol extracts of *C. pacificum* organs were dissolved in 70% methanol at a concentration of (0.5, 1, 1.5, 2, 2.5, 3 mg/mL) where 200 µL of each test solution added to 6 mL DDPH solution. The DPPH was prepared at a concentration of 0.004 % in methanol (HPLC grade). Blank samples were run using 99.9% (absolute) methanol. The mixture was left for 30 min at room temperature then the absorbance was measured spectrophotometrically against a blank at 517 nm. Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] and silymarin were used as positive controls at a concentration of (0.05, 0.07, 0.09, 0.11, 0.13 mg/ml), and (0.5, 1, 1.5, 2, 2.5, 3 mg/ml), respectively. Free radical scavenging activity is expressed as the concentration of extract inhibiting DPPH formation by 50% relative to methanol (IC_{50} value).

Determination of polyphenolic content

Aliquots (2 ml, each) of 50% methanolic extracts and standard solution of gallic acid (0.008, 0.016, 0.024, 0.032, 0.04, 0.048, and 0.056 mg/mL) was added to 1.5 mL of Folin-Ciocalteu reagent and 4 ml of 20% sodium carbonate solution, then the solution was diluted to 25 ml with distilled water. The absorbance of the resulting solution was measured, after 30 min at λ_{max} 765 nm, against a blank prepared at the same time, using 2 ml of distilled water, instead of the tested solution. For each concentration, triplicate experiments were carried out. The results were expressed as µg of gallic acid equivalents/ mg of extract (GAE)

Determination of total flavonoids content

Aliquots (0.5 ml, each) of 80% methanol extracts and standard solution of quercetin (0.01, 0.02, 0.04, 0.08 and 0.1 mg/mL) was added to 0.1 Ml of 10% aluminium chloride, 0.1 mL of 1M potassium acetate aqueous solution and 2.8 mL of distilled water. The solution was well mixed and the absorbance was measured spectrophotometrically at 415 nm against blank. Total flavonoids content was expressed as mg quercetin equivalent (QE)/g plant extract. Each measurement was performed in triplicate.

Extraction and isolation

Fresh flowers (3.5 kg) was macerated in 70% ethanol till exhaustion. The ethanolic extract was evaporated under vacuum to yield (95 g). The residue was suspended in water and partitioned successively with petroleum ether, chloroform followed by ethyl acetate and *n*-butanol saturated with water. The solvents were then evaporated under reduced pressure to give different fractions. The EtOAc residue (10.5 g) was subjected to silica gel CC and eluted by gradient addition of $CHCl_3$, EtOAc and MeOH to yield fractions I & II. Fr. I (350 mg) was purified by sephadex LH-20 using MeOH-H_2O (1:1 v/v) to yield compound 1. Fraction II was purified by sephadex LH-20 using MeOH-H_2O (1:1 v/v) and further purified by rechromatography on a silica gel column (13 cm L×1 cm D). Gradient elution was performed using $CHCl_3$-MeOH (95:5 v/v) with increasing MeOH percentage by 1% till reaching 10% MeOH to yield compound 2. *n*-BuOH fraction was subjected to VLC column eluted with $CHCl_3$ and increasing the polarity by 5% increments with EtOAc, MeOH and H_2O till concentration 20% to yield fractions A & B. Fraction A (700 mg) was subjected to purification on sephadex LH-20 MeOH-H_2O (1:1 v/v), to yield compound 3. Fraction B (2.7 g) was chromatographed on sephadex LH-20 column using MeOH-H_2O (1:1 v/v) followed by further purification on C_{18}–reversed phase column to yield compound 4.

RESULTS AND DISCUSSION

Estimation of free radical scavenging activity

Free radical scavenging activity of *C. pacificum* Nakai organs was assessed using a modified 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay(14). Results (Table 1 & Figure 1) revealed that the flowers methanol extract is the most effective in inhibiting DPPH (IC_{50}, 1.2 mg/ml), and even more potent than silymarn (IC_{50}, 1.84 mg/ml), whereas roots extract was the least effective (IC_{50} value of 1.9 mg/ml).

Table 1: Free radical scavenging activity of methanol extracts of *C. pacificum* organs

Organ	DPPH inhibition IC_{50} (mg/mL)
Flower	1.2 ± 0.05
Aerial part	1.69 ± 0.03
Root	1.9 ± 0.04
Silymarin	1.84 ± 0.07
Trolox	0.09 ± 0.001

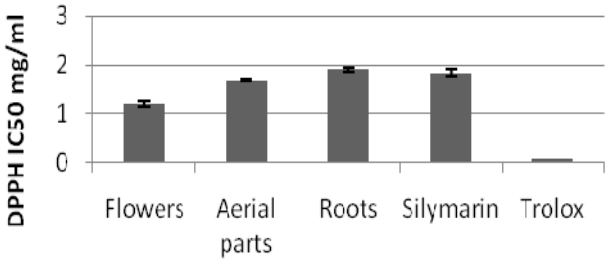


Figure 1: Histogram showing free radical scavenging activity of *C. pacificum* organs methanol extract

Estimation of hepatoprotective activity

This is the first report on the *in- vitro* hepatoprotective effect of *C. pacificum* parts using a cell culture model consisting of a human hepatoma cell line (Huh7) that was treated with CCl_4 to induce hepatocyte damage. The protective effect of the tested samples was evaluated based on the changes in aspartate aminotransferase (AST), alanine transaminase (ALT), superoxide dismutase (SOD) activities and liver glutathione (GSH) level before and after exposure of the cells to CCl_4(15). Exposure of Huh7 cells to CCl_4 significantly ($p<0.001$) increased AST, ALT, decreased SOD activities to 40.45 ±3.15, 36.41 ±2.84 and 168.48 ±3.7 U/ml respectively and decreased GSH level to 2.38 ±0.37 mg/dl in comparison to control untreated cells. Pretreatment of cells with different concentrations of silymarin (10,100,1000 µg/ml) significantly ($p<0.001$) ameliorated CCl_4-induced damage through decreasing AST, ALT activities and increasing SOD activity. At the same time silymarin increased GSH level (Table 2 & Figure 2). All tested samples significantly reduced elevated AST and ALT activities ($p<0.001$) as compared to CCl_4 intoxicated group. Extracts of flowers (100 µg/ml), aerial parts (100 & 1000 µg/ml) and roots (100 µg/ml) significantly ($p<0.001$) reduced AST and ALT levels compared with CCl_4 treated group. Ethanol extract of the flowers (100 & 1000 µg/ml) exhibited a significant increase ($p<0.001$) in SOD activity (237.18 & 371.77 U/ml respectively) (Table 2 & Figure 2). GSH reduced level following CCl_4 intoxication was significantly ($p<0.001$) restored by pretreatment with all tested samples (Table 2 & Figure 2). The extracts of flowers (10, 100 & 1000 µg/ml), and aerial parts (10 & 1000 µg/ml) increased GSH level to 22.64, 25.42, 25.75, 21.71 & 22.19 mg/dl respectively.The forementioned results revealed that ethanolic extract of the flowers demonstrated the highest potency regarding SOD and GSH enzymes at all concentration levels, and showed comparable activity to the ethanolic extract of the aerial parts & roots regarding AST and ALT levels, followed by that of aerial parts, while the extract of the roots was the least effective in normalizing the CCl_4-induced damage on Huh7 cell lines. The same human hepatoma cell line (Huh7) model was adopted to estimate the hepatoprotective potential of petroleum ether (PE), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and remaining aqueous (H_2O) fractions of the flowers ethanol extract as the most potent organ (Table 3& Figure 3).

Table 2: Protective effect of *C. pacificum* organs ethanol extract relative to CCl_4-induced damage on the Huh7 human hepatoma cell line

Group	Conc µg/ml	AST U/ml	AST %change[c]	ALT U/ml	ALT %change[c]	SOD U/ml	SOD % change[c]	GSH mg/dl	GSH % change[c]
Normal	-	16.34±0.19	--	27.29±1.15	--	317.83±8.1	--	13.48±0.90	--
CCl_4	-	40.45#±3.15	+147.531	36.41#±2.84	+33.39	168.48#±3.7	-46.99	2.38#±0.37	-82.37
Sil	**10**	21.46*±3.46	-46.95	28.32*±3.11	-22.23	225.92*±1.59	+34.09	28.13*±3.2	+1083.27
	100	16.55*±0.43	-59.10	24.89*±0.39	-31.64	235.78*±1.18	+39.95	30.93*±2.5	+1201.03
	1000	17.92*±1.61	-55.71	25.11*±0.12	-31.01	292*±0.70	+73.23	38.50*±2.78	+1519.63
Flower	**10**	26.91*±0.35	-33.47	23.12*±0.32	-36.49	211.02*±7.74	+25.25	22.64*±0.53	+852.34
	100	22.51*±1.23	-44.36	20.26*±1.11	-44.36	237.18*±6.5	+40.78	25.42*±1.74	+969.18
	1000	24.58*±0.47	-39.24	22.52*±0.42	-38.14	371.77*±2.59	+120.67	25.75*±0.42	+983.19
AP	**10**	24.65*±0.35	-39.05	22.19*±0.32	-39.05	117.01±4.64	-30.54	21.71*±0.92	+813.08
	100	18.43*±0.36	-54.45	16.58*±0.31	-54.45	128.66±1.01	-23.63	18.73*±0.19	+687.85
	1000	22.60*±1.41	-44.13	20.34*±1.27	-44.13	182.44±0.46	+8.29	22.19*±0.72	+833.64
Root	**10**	28.64*±1.19	-29.20	25.78*±1.07	-29.20	107.33±4.24	-36.29	18.86*±0.37	+693.46
	100	22.33*±2.33	-44.82	20.09*±2.09	-44.82	116.71±4.15	-30.73	17.37*±0.10	+630.84
	1000	23.61*±0.13	-41.64	21.25*±0.12	-41.64	103.60±2.24	-38.50	17.86*±0.34	+651.40

a-Results are expressed as mean ± S.D.; b-Sil =Silymarin; AP=Aerial parts

c-(+) represents percentage of increase and (-) represents percentage of decrease in each value when compared to either normal or CCl_4

*Statistically significant difference from CCl_4 group at $p < 0.001$; # Statistically significant difference from normal group at $p < 0.001$

Statistical analysis was performed using one-way ANOVA followed by Tukey test

Table 3: Protective effect of *C. pacificum* flowers fractions against CCl_4-induced damage on the Huh7 human hepatoma cell line

	Conc	AST		ALT		SOD		GSH	
	µg/ml	U/ml	%change[c]	U/ml	%change[c]	U/ml	%change[c]	mg/dl	%change[c]
Normal	-	13.03±0.93	--	11.58±1.45	--	327.37±5.07	--	9.20±0.52	--
CCl_4	-	24.18#±2.1	85.57	26.41#±1.86	128.06	181.37#±2.05	-44.59	2.38#±0.18	-74.13
Sil	**10**	9.78*±0.32	-59.55	19.89*±1.69	-24.68	231.33*±3.54	27.54	11.24*±0.12	372.27
	100	9.25*±0.09	-61.75	16.38*±0.47	-37.97	275.01*±5.13	51.62	16.73*±1.33	602.94
	1000	8.1*±1.03	-66.50	13.30*±0.43	-49.64	308.92*±6.18	70.33	22.80*±0.03	857.98
PE	**10**	15.35*±0.25	-36.52	22.67*±0.32	-14.16	179.04±8.18	-1.28	13.26*±0.09	457.14
	100	14.25*±0.17	-41.07	21.92*±0.65	-17.00	184.67±8.18	1.8	16.21*±0.15	581.09
	1000	14.15*±0.04	-41.48	16.07*±1.73	-39.15	187.33±6.18	3.28	18.60*±0.07	681.51
$CHCL_3$	**10**	15.21*±0.05	-37.09	20.41*±0.65	-22.71	190.67±1.31	5.13	11.26*±0.38	373.11
	100	14.51*±0.25	-39.99	15.13*±0.32	-42.71	203.67±5.01	12.29	12.51*±0.10	425.63
	1000	14.18*±0.07	-41.35	13.18*±1.08	-50.09	205.75±2.75	13.44	14.23*±0.37	497.89
EtOAc	**10**	14.88*±0.15	-38.46	16.01*±0.21	-39.37	271.4*±5.09	49.64	12.24*±0.15	414.28
	100	14.2*±0.05	-41.27	11.42*±0.43	-56.75	276.7*±1.31	52.56	16.53*±0.16	594.54
	1000	11.2*±0.51	-53.68	6.45*±0.25	-75.57	339.28*±5.27	87.07	19.06*±0.17	700.84
***n*-BuOH**	**10**	14.53*±0.50	-39.90	17.27*±0.93	-34.60	267.8*±2.25	47.65	12.26*±0.09	415.12
	100	14.21*±0.28	-41.23	13.68*±0.87	-48.20	276.7*±2.19	52.56	18.4 *±0.10	673.10
	1000	14.13*±0.15	-41.56	11.02*±0.07	-58.27	328.5*±5.53	81.12	22.06*±0.26	826.89
H_2O	**10**	14.89*±0.67	-38.42	17.08*±0.43	-35.32	190.53±1.27	5.05	14.71*±0.05	518.06
	100	15.33*±0.05	-36.60	14.12*±1.08	-46.53	200.43±3.57	10.50	15.01*±0.06	530.67
	1000	14.91*±0.07	-38.33	11.61*±0.10	-56.03	204.13±3.75	12.54	17.55*±0.05	637.39

a-Results are expressed as mean ± S.D.; b-Sil=Silymarin; PE=Petroleum ether; H_2O=Remaining water fraction
$c^{(+)}$ represents percentage of increase and $^{(-)}$ represents percentage of decrease in each value when compared to either normal or CCl_4
* Statistically significant difference from CCl_4 group at $p < 0.001$; # Statistically significant difference from normal group at $p < 0.001$
Statistical analysis was performed using one-way ANOVA followed by Tukey test

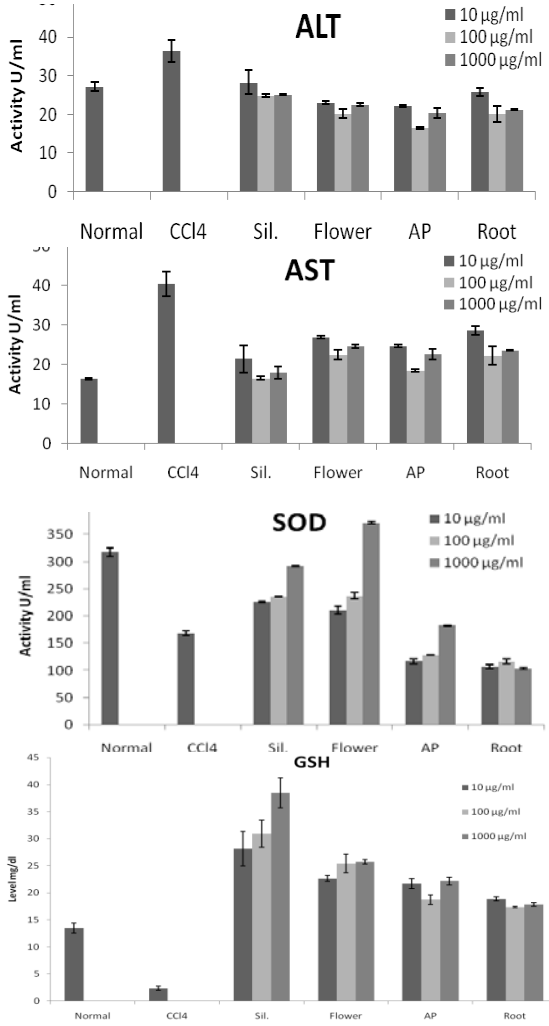


Figure 2: Protective effect of ethanol extract of *C. pacificum* parts relative to CCl_4

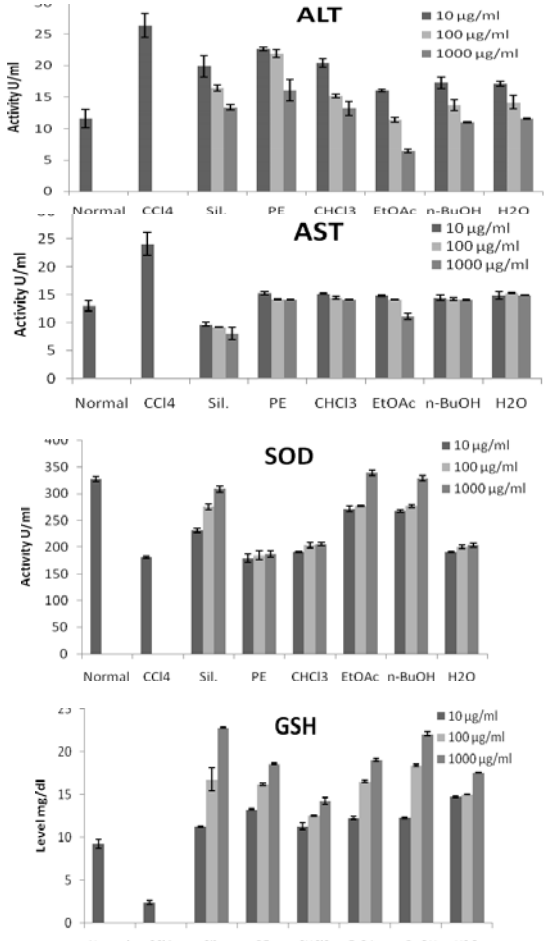


Figure. 3: Protective effect of *C. pacificum* flowers fractions relative to CCl_4 on the activity of AST, ALT & SOD and level of GSH using *in-vitro* assay on human hepatoma cell line (Huh7). **Sil., Silymarin; PE, petroleum ether fraction; H_2O, remaining water fraction.**

EtOAC and *n*-BuOH (100 & 1000 µg/ml) were the most effective fractions ($p<0.001$) in neutralizing the reduced SOD activity, GSH level and the elevated ALT level. Furthermore, EtOAC and *n*-BuOH fractions demonstrated higher potency than silymarin in normalizing ALT and SOD activities. Moreover, *n*-BuOH fraction was more effective in neutralizing GSH levels compared to silymarin.

Determination of total polyphenol content

Total polyphenol content was determined spectrophotometrically (Figure 4) according to the Folin-Ciocalteu colorimetric method (16). Flowers (53.65 ± 1.15) was the most enriched part in the total polyphenols followed by aerial parts (42.01 ± 0.91) whereas roots extract showed the least content (27.99 ±0.62 mg/g plant extract).

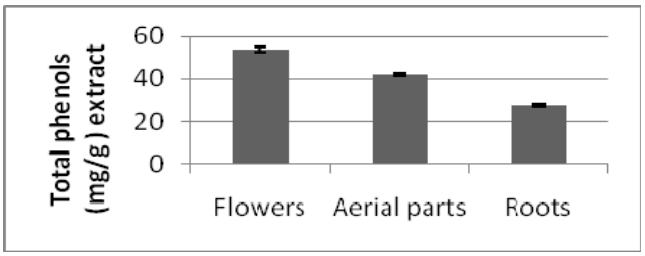


Figure 4: Total polyphenol content of *C. pacificum* parts.

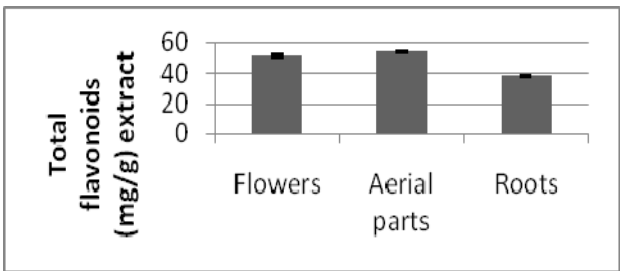


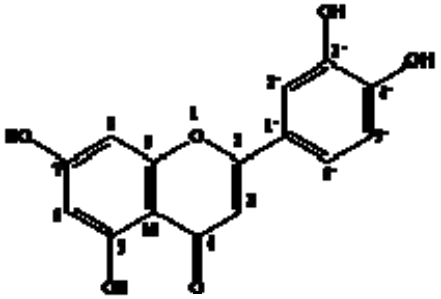
Figure 5: Total flavonoids (TF) content of *C. pacificum* parts.

Determination of total flavonoids content

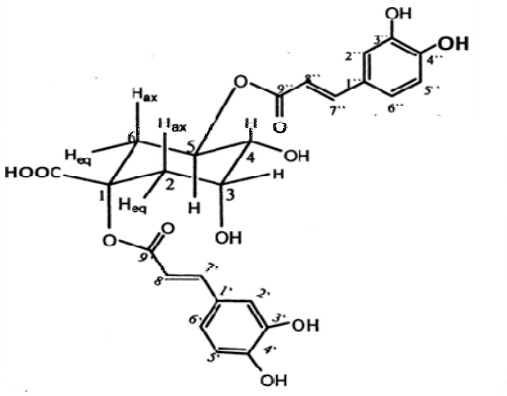
Total flavonoids (TF) content was measured using $AlCl_3$ colourimetric assay (17). Results (Figure 5) revealed that TF expressed as mg quercetin /g plant extract was comparable in the flowers and aerial parts reaching 51.52 ± 1.2 & 54.02 ± 0.7mg/g extract, respectively whereas the roots showed the lowest TF content (38.28± 0.52 mg/g extract). Correlation appears to exist between total polyphenols and flavonoids content and the investigated activities for flowers, aerial parts and roots samples. Flowers demonstrating strong antioxidant & hepatoprotective effects were enriched in total polyphenols and flavonoids with an average concentration of 53.65 & 51.52 mg/g plant extract respectively.

Isolation and characterization of phytochemicals from flowers fractions

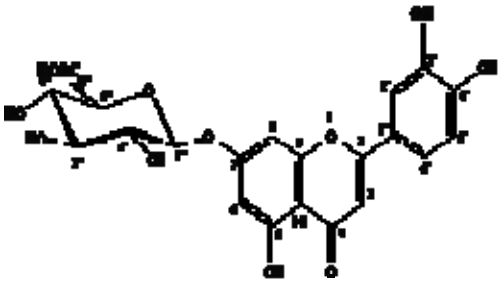
As EtOAc and *n*-BuOH fractions exhibited the highest hepatoprotective effect, these two fractions were selected for isolation of phytochemicals. Luteolin (1) and 1,5-di-O-caffeoylquinic acid (2) were isolated from EtOAc fraction, while luteolin-7-O-β-glucuronide (3) and luteolin-7-O-rutinoside were isolated from *n*-BuOH fraction (Figure 6).



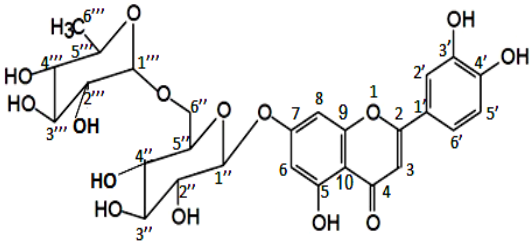
Luteolin (1)



1,5-Di-O-caffoeylquinic acid (2)



Luteolin-7-*O*-β-glucuronide (3)



Luteolin-7-*O*-rutinoside (4)

Figure 6: Chemical structures of compounds isolated from *C, pacificum* flowers

The structures of the isolated compounds (Figure 6) were established by comparing their UV, ^1H and ^{13}C NMR spectroscopic data with those in the literature and confirmed through co-chromatography with authentic samples. Compound 1 (Luteolin, 70 mg) was isolated as yellow powder, displayed a molecular formula of $C_{15}H_9O_6$ derived from its HR-ESI-MS data (*m/z* 285.0409 $[M-H]^-$). The ^1H NMR spectral data showed the characteristic signals of a luteolin nucleus; three protons of ring-B, at δ

6.9 and 7. 4 (each d, J =8 Hz) for H-5' and H-6', respectively and H-2' at δ 7. 4 (1H, s), in addition to a pair of meta-coupled aromatic protons at δ 6.2 & 6.41 (each d, J = 2 Hz) for H-6 and H-8 respectively whereas H-3 protons appeared at δ 6.57 (1H, s). Published data(18) and co-chromatography against in-house authentic standard confirmed its identification. Compound 2 (1,5-di-O-caffeoylquinic acid, 15 mg) was isolated as white powder. Its ^{1}H NMR spectral data showed the characteristic signals for two caffeic acid molecules(19-20). Two pairs of *trans*-olefinic protons, the first at δ 7.44 & 6.23 ppm, (1H J= 15.9 & 15.6 Hz) assigned to H-7' and H-8' and second at δ 7.37 & 6.16 ppm (1H, J= 15.6 & 15.9 Hz) assigned to H-7" and H-8" respectively. A pair of three protons, exhibiting two ABX systems was displayed. Two doublets at δ 7.03 & 7.07 ppm (J= 1.5 & 1.8 Hz) assigned to H-2" and H-2', respectively and a multiplet at δ 6.76 ppm was assigned to H-5" and H-5'. In addition, a multiplet at δ 6.95 ppm, integrated as two protons was assigned to H-6" and H-6'. It also showed the characteristic signals of a quinic acid molecule(20-21). Two multiplets at δ 1.83 & 2.39 ppm and two other multiplets at δ 2.20 & 2.50 ppm assigned to H-6 axial & H-6 equatorial and H-2 axial & H-2 equatorial, respectively. Three multiplets at δ 4.04, 5.27 and 3.53 ppm assigned to H-3, H-5 and H-4, respectively. It was obvious that only H-5 was downfield shifted, indicating the presence of a caffeoyl moiety at position 5 of the quinic acid molecule. On the other hand, the signals of H-3 and H-4 were not significantly desheilded, suggesting that the other caffeoyl moiety is attached to C-1 of the quinic acid (22-23). Further evidence for esterification at C-1 was provided by the signals for the two protons H-2 axial and H-2 equatorial, which lost their magnetic equivalence and appeared at δ 2.20 & 2.50, respectively(24).

Compound 3 (luteolin-7-O-β-glucuronide, 10 mg) was isolated as yellow powder, its UV spectroscopic data [λ_{max} 258, 267, 348] indicated its flavone nature(25-26). It displayed a molecular formula of $C_{21}H_{17}O_{12}$ derived from its HR-ESI-MS data (*m/z* 461.0815 $[M-H]^-$), and further yielding an ion fragment at *m/z* 285.0461 $[M-H-176]^-$ of luteolin due to the loss of one glucuronide moiety, suggesting for the presence of luteolin glucuronide(26). Analysis of its ^{1}H NMR spectrum and co-chromatography against in-house authentic standard confirmed our interpretation. Compound 4 (luteolin-7-O-rutinoside, 40 mg) was isolated as yellow powder, its ^{1}H NMR spectral data showed the characteristic signals of luteolin nucleus as described under compound 1. The sugar units were identified as rhamnoglucoside based on ^{13}C NMR spectrum. The presence of the anomeric proton at δ 4.91 (J= 7.70 Hz) indicated β-configuration of glucose(27). The methyl group of rhamnose was displayed at δ 1.09 (J=6 Hz). The anomeric proton of rhamnose unit resonate at δ 4.62 as singlet, confirms its α- configuration. The direct evidence for the sugars sequence and their linkage sites was determined from the results of HMBC experiment which showed unequivocal correlation peaks between H-1glc-C-7 & H-1rha-C-6glc.

ACKNOWLEDGMENT

The authors are grateful for Dr. Ashraf B. Abdel-Naim professor of pharmacology Ain Shams University, Cairo, Egypt for performing the hepatoprotective study.

CONCLUSION

The protective effect of *C. pacificum* flowers is at least partly attributed to its antioxidant activity as evidenced by the reduction in GSH level, the increase in SOD activity and confirmed by the significant radical scavenging activity. The identified compounds possesses hepatoprotective potential(4,5,22,28), furthermore, they are similar to the active compounds of artichoke, particularly 1,5-di-O-caffeoylquinic acid (cynarin), luteolin-7-O-rutinoside (scolymoside) and luteolin (29-31). Our results suggest that *C. pacificum* contained biologically active constituents and need further investigations supporting its use as hepatoprotective agent.

REFERENCES

1- Fraschini F, Demartini G, Esposti D. Pharmacology of silymarin. Clin Drug Invest 2002, 22,51-65.
2- Medina J, Moreno-Otero R. Pathophysiological basis for antioxidant therapy in chronic liver disease. Drugs 2005,65, 2445-61.
3- Yang L, Wua D, Cederbaum AI. CYP2E1, oxidative stress and MAPK signaling pathways in alcohol-induced hepatotoxicity. J Biochem Pharmacol Res 2014,2,74-90.
4- Meng L, Lozano YF, Gaydou EM, Li B. Antioxidant activities of polyphenols extracted from *Perilla frutescens* varieties. Molecules 2009,14,133-40.
5- Sugawara T, Igarashi K. Identification of major flavonoids in petals of edible *Chrysanthemum* flowers and their suppressive effect on carbon tetrachloride-induced liver injury in mice. Food Sci Technol Res 2009,15,:499–506.
6- Fraisse D, Felgines C, Texier O, Lamaison JL. Caffeoyl derivatives: major antioxidant compounds of some wild herbs of the Asteraceae family. Food Nutr Sci 2011,2,181-92.
7- Abdallah HM, Ezzat SM, El Dine RS, Abdel-Sattar E, Abdel-Naim AB. Protective effect of *Echinops galalensis* against CCl_4-induced injury on the human hepatoma cell line (Huh7). Phytochemistry Lett 2013,6,73-78.
8- Kim HJ, Lee YS. Identification of new dicaffeoylquinic acids from *Chrysanthemum morifolium* and their antioxidant activities. Planta Med 2005,71,871-6.
9- Clifford MN, Wu W, Kirkpatrick J, Kuhnert N. Profiling the chlorogenic acid and other caffeic acid derivatives of herbal *Chrysanthemum* by LC-MSn. J Agric Food Chem 2007,55,929-36.
10- Lai JP, Lim YH, Su J, Shen HM, Ong CN. Identification and characterization of major flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD-APCI/MS. J Chromatogr B 2007,848,215-25.
11- WangYJ, Yang XW, Guo QS. Studies on chemical constituents in Huangjuhua (flowers of *Chrysanthemum morifolium*). China J Chinese Materia Med 2008,33,526-530.
12- Wu LY, Gao HZ, Wang XL, Ye JH, Lu JL, Liang YR. Analysis of chemical composition of *Chrysanthemum indicum* flowers by GC/MS and HPLC. J Med Plants Res 2010,4, 421-6.
13- Nermeen FF, Mohamed AF, Enas HA, Shadia MA, El-Sayeda AK. Metabolites profiling of *Chrysanthemum pacificum* Nakai parts using UPLC-PDA-MS coupled to chemometrics. N. Prod. Res 2015 (under publication).
14- Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992,40, 945-8.
15- Torres-Gonzalez L, Munoz-Espinosa LE, Rivas-Estilla AM, Trujillo-Murillo K, Salazar-Aranda R., De Torres NW, Cordero-Perez P. Protective effect of four Mexican plants against CCl_4 induced damage on the Huh 7 human hepatoma cell line. An Hepato 2011, 10,73-9.

16- Saboo S, Tapadiya R., Khadabadi SS , Deokate UA. In vitro antioxidant activity and total phenolic, flavonoid contents of the crude extracts of *Pterospermum acerifolium* wild leaves (Sterculiaceae). J Chem Pharm Res 2010,2,417-23
17- Mandal B, Madan S. Preliminary phytochemical screening and evaluation of free radical scavenging activity of *Stevia rebaudiana* Bertoni from different geographical sources. J Pharmacogn Phytochem 2013,2,14-9.
18- Cakir A, Mavi A, Kazaz C, Yildirim A, Kufrevioglu OI. Antioxidant activities of the extracts and components of *Teucrium orientale* L. var. *orientale*. Turk J Chem 2006,30, 483-94.
19- Carnat A, Heitz A, Fraisse D, Carnat AP, Lamaison JL. Major dicaffeoylquinic acids from *Artemisia vulgaris*. Fitoterapia 2000;71:587-9.
20- Papajewski S., Vogler B., Conrad J., Klaiber I., Roos G., Walter CU., Sussmuth R., Kraus W. Isolation from *Cussonia barteri* of 1'-O-chlorogenoylchlorogenic acid and 1'-O-chlorogenoylneochlorogenic acid, a new type of quinic acid esters. Planta Med 2001,67, 732-6.
21- Tolonen A, Joutsamo T, Mattlla S, Kamarainen T, Jalonen J. Identification of isomeric dicaffeoylquinic acids from *Eleutherococcus senticosus* using HPLC-ESI/TOF/MS and ^{1}H-NMR methods. Phytochem Anal 2002;13:316–28.
22- Domitrovic R, Jakovac H, Milin C, Radosevic-Stasic B. Dose- and time-dependent effects of luteolin on carbon tetrachloride-induced hepatotoxicity in mice. Exp Toxicol Pathol 2009,61,581-9.
23- Elsayed SM, Nazif NM, Hassan RA, Hassanein HD, Elkholy YM, Gomaa NS, Shahat AA. Chemical and biological constituents from the leaf extracts of the wild artichoke (*Cynara cornigera*). Internat J Pharm Sci 2012;4:396-400.
24- Merfort I. Caffeoylquinic acids from flowers of *Arnica montana* and *Arnica Chamissonis.* Phytochtmistry 1992,31,2111-3.
25- Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids 1970. New York, Heidelberg, Berlin, Springer-Verlag.
26- Krenn L, Miron A, Pemp E, PetrU, Kopp B. Flavonoids from *Achillea nobilis* L. Z Naturforsch C 2003,58,11-6.
27- Agrawal PK. NMR-spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 1992,31,3307-30.
28- Lee MJ, Lee HS, Park SD, Moon HI, Park WH. Protective effects of luteolin-7-*O*-beta-D-glucuronide methyl ester from the ethyl acetate fraction of *Lycopi herba* against pro-oxidant reactive species and low-density lipoprotein peroxidation. J Enzyme Inhib Med Chem 2010,25,702-7.
29- Wang M, Simon JE, Aviles IF, He K, Zheng QY, Tadmor Y. Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). Journal of Agricultural and Food Chem 2003,51,601-8.
30- Mehmetcik G, Ozdemirler G, Kocak-Toker N, Cevikbas U, Uysal M. Effect of pretreatment with artichoke extract on carbon tetrachloride-induced liver injury and oxidative stress. Exper Toxicol Pathol 2008, 60,475-80.
31- Farag MA, El-Ahmady SH, Elian FS, Wessjohann LA. Metabolomics driven analysis of artichoke leaf and its commercial products via UHPLC-q-TOF-MS and chemometrics. Phytochemistry 2013,95,177-87.