Original Article

Isolation, Structure Elucidation of Ferulic and Coumaric acids from *Fortunella japonica* Swingle leaves and their Structure Antioxidant activity relationship

Heba Abd-Elhady El-gizawy[1] and Mohammed Abdalla Hussein[2*]

[1]Department of Pharmacognosy, Faculty of Pharmacy, October 6th University, Sixth of October City, EGYPT.
[2]Department of Biochemistry, Faculty of Pharmacy, October 6th University, Sixth of October City, EGYPT.

ABSTRACT

Objective: The present study deals with isolation, structure elucidation of ferulic and coumaric acids from *Fortunella japonica* Swingle leaves and their structure antioxidant activity relationship. Structural analysis was conducted using UV, IR, mass and Nuclear Magentic Resonance (NMR) spectroscopy. **Methodology:** In particular, completely assigned ^{1}H and ^{13}C-NMR data are presented. Also, the present study was extended to investigate the hepatoprotective activity of isolated compounds against paracetamol-induced toxicity in freshly isolated rat hepatocytes model. Freshly isolated rat hepatocytes were exposed to paracetamol (5 mM) along with/without various concentrations of the isolated compounds (40–80μg/ml). **Results and Discussion:** Exposure of isolated hepatocytes to paracetmol resulted in lipid hydroperoxides formation, depletion in protein thiols, superoxide dismutase (SOD), succinate dehydrogenase (SDH), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) levels as well as elevation of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). The isolated coumaric and ferulic acids have been found to efficiently inhibit paracetamol-induced biochemical alterations, namely oxidative stress biomarkers and protein oxidation. It also significantly prevented paracetamol-induced loss in the activity of antioxidant enzyme and the important endogenous antioxidant glutathione. **Conclusion:** The study suggests that coumaric and ferulic acids can act as an antioxidant and hepatoprotective in physiological systems.

Key words: *Fortunella japonica***,** Coumaric acid, Ferulic acid, Hepatocytes, Antioxidant, Hepatoprotective.

Correspondence :

Mohammed Abdalla Hussein, Ph.D.,
Department of Biochemistry,
Faculty of Pharmacy, October 6th University, 6th of October City, EGYPT.
Phone no.:+2(0122) 4832580; fax: +2 (02) 38353270.

E-mail: Prof.husseinma@o6u.edu.eg

INTRODUCTION

Leaves of *Fortunella japonica* Swingle or kumquat are slow- growing evergreen shrubs, sometimes bearing small thorns, they belong to family Rutaceae, either forming the genus *Fortunella* or placed within genus *Citrus*. The leaves are dark glossy green, the tree producing edible golden yellow colored fruits, the plant is native to south Asia, they have long been cultivated in Japan and Taiwan, they were introduced to Europe in 1846 by Robert Fortune.[1] The leaves and fruits of the *Fortunella* species have been used in folk medicine in China and recently more attempts have been made to study the pharmacological activities of some bioactive compounds isolated from kumquat, the peel of the fruit has been extensively studied.[2] Phenolic composition and antioxidant characteristics of kumquat were only confined to the peel content of the fruits.[2]

The most widely distributed polyphenolic compounds in plant tissues are hydroxycinnamic acids. Some of the most common naturally occurring HCAs are *p*-coumaric acid, ferulic acid, sinapic acid, and caffeic acid. Their biological effects are strongly dependent on the number and position of hydroxyl groups.[3]

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital.[4] Free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals.[5] A role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and cardiovascular diseases.[6]

Ferulic and coumaric acids (Figure 1) are an important biological and structural component of the plant cell wall.[7] Due to their ability to stop radical chain reactions by resonance followed by polymerization, ferulic acid offers protection against UV-radiation and is responsible for cross-linking polysaccharides and other cell wall polymers.[8] The antioxidant activity of ferulic and coumaric acids has been reported for scavenging NO, O_2^- and –OH.[9]

Ferulic acid also has a hepatoprotective effect against toxicity induced *in vivo* by carbon tetrachloride.[10]

Paracetamol is a widely used analgesic and antipyretic medication.[11] Paracetamol causes acute hepatic necrosis in rats and other animal species.[12] However, when given in large single-dose ingestions, paracetamol can induce liver, kidney, and other organ damages in both humans and animals.[13] At therapeutic doses, paracetamol mainly undergoes glucuronidation and sulfation in the liver.[14] On the other hand, at over dosages, paracetamol is metabolized by the cytochrome P450 (CYP450) oxidative system that is mainly localized in the liver endoplasmic reticulum, and generates N-acetyl-p-benzoquinone-imine (NAPQI).[15] This metabolite depletes hepatic glutathione and then binds covalently to intracellular proteins, including mitochondrial proteins.[15] This situation leads to the formation of reactive oxygen and nitrogen species, and initiates lipid peroxidation that eventually results in destruction, necrosis, or apoptosis of the liver cells.[14]

In continuation of our interested research program in the isolation and therapeutic evaluation of natural products,[16,17] the present study report here in, a facile route to isolate ferulic and coumaric acids from *Fortunella japonica* Swingle leaves and investigate their *in-vitro* hepatoprotective effects against paracetamol-induced oxidative damages in isolated hepatocytes.
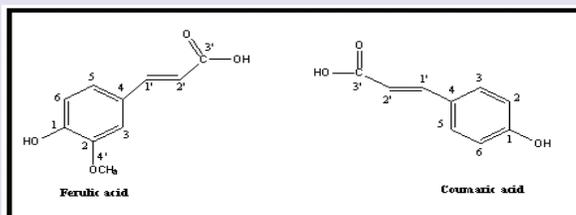
Figure 1: chemical structure of ferulic and coumaric acids.

MATERIALS AND METHODS

Chemicals and Reagents

- All chemicals were purchased from Sigma Chemicals Co., St. Lious, MO, USA.

  Diaion HP-20 Resin, SUPELCO, Bellefonte, PA,USA.

- *Thin Layer Chromatography (TLC)*

  TLC precoated TLC plates [silica gel 60 GF254 (20×20 cm) and (10×20 cm), Merck, Germany] and sheets of Whatman No. 1 filter paper, (El Masryia Co.) for P.C. were used as stationary phase.

Plant material

Leaves of *Fortunella japonica* Swingle were collected in November from private farm in Giza governorate, were identified by Madam Treas, plant taxonomist, El Orman graden, voucher specimen was deposited at the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, October 6 University, No. (A11-2014).

Apparatus

- *Column chromatography*

  Glass columns of different sizes were packed with the adsorbent diaion HP-20.

- Melting points were determined on Gallenkamp melting point appa-ratus and are uncorrected. The infrared (IR) spectra were recorded on Shimadzu MR 470 infrared spectrophotometer using the KBr pellets. ^{1}H- and ^{13}C-NMR spectra were recorded in DMSO-d_6 and CD$_3$OD, respectively, on a Varian EM 500 (^{1}H NMR at 400MHz) and (^{13}C NMR at 100MHz). The chemical shifts are reported in part per million (δ ppm) downfield from internal tetramethylsilane (TMS). Mass spectra were run using HP Model MS-5988.

- **UV-visible spectrophotometer**, Shimadzu UV 240 (P/N 204-58000) was used for recording UV spectra and measuring absorbance in UV range. **Ultraviolet lamp** for localization of spots on paper and thin layer chromatography.

Plant extraction

The leaves (1.5 Kg) of *Fortunella japonica* Swingle were shade dried then mechanically powdered and subjected to extraction by percolator, using methanol , the extract was concentrated to dryness under reduced pres-sure and controlled temperature by distillation in *vacuum* to yield a dark green colored mass, which was dissolved in 500 ml of water and extracted eight times with 500 ml of n- hexane, then extracted five times with 500 ml of ethyl acetate and finally three times with 500 ml of n-butanol, after removing the solvents, the n-butanol fraction afforded 22 g.

Investigation of the n-butanol fraction

The n-butanol fraction (12 g) was loaded on a column chromatography (100 L x 4.5 ID cm), packed with ion exchange resin, Diaion HP-20 as a stationary phase. Elution started with distilled water followed by mixtures of (water/ methanol) by stepwise addition of 10% of methanol till methanol 100%. Twenty ml fractions were collected and checked for purity by TLC (with developing system composed of methylene chloride and methanol of different proportions) and paper chromatography using solvent system S1-S2; (S1: n-BuOH-AcOH- H$_2$O, v/v/v: 4:1:5), (S2: AcOH – H$_2$O: 15:100). Similar fractions were pooled together and subjected to further purification.

Fraction I: eluted by MeOH - H$_2$O (20:80), spotted on TLC was found to contain one spot, the solvent was evaporated at low temperature under reduced pressure to yield compound C1 (R$_f$ =0.66 in S1 & 0.69 in S2) on PC. Recrystallization from methanol afforded compound C1 as buff powder (40 mg).

Fraction II: eluted by MeOH-H$_2$O (30: 70), spotted on TLC was found to contain one spot, the solvent was evaporated at low temperature under reduced pressure to yield compound C2 (R$_f$ =0.54 in S1 & 0.66 in S2) on PC. Recrystallization from methanol afforded compound C2 as white crystal (50 mg).

Compounds were identified based on chromatographic properties and confirmed by UV, IR, mass, ^{1}H- and ^{13}C-NMR spectral data.

Identification of compound C1

Compound C1 was obtained as buff powder (40 mg), mass spectrum showed a molecular ion and base peak at 164 m/z with other signifi-cant beaks at 147(53%), 119(42%), 107(66%), 91(33%), 77(45%) and 65(72%) using EI mode. The chemical formula of C1 is (C$_9$H$_8$O$_3$). The IR (KBr, cm^{-1}) spectrum with the peaks at 3390 cm^{-1} (carboxylic acid O-H stretching), 3070 and 2916 cm^{-1} (CH-arom), 2819 cm^{-1} (CH-aliph.), 1672 cm^{-1} (carboxylic acid C=O stretching), 1250 cm^{-1} (carboxylic acid C-O stretching) and 1520; 1600 cm^{-1} (aromatic C=C).

The UV, IR, mass, 1H- and ^{13}C-NMR spectral data as well as co-chroma-tography with standard confirms the skeleton of ρ-coumaric acid.

Identification of compound C2

Compound C2 was obtained as yellow crystals (50 mg), mass spectrum showed a molecular ion and base peak at 194 m/z with other significant peaks at 179(27%), 161(16%), 105(8%), 92(6%), 77(26%) and 51(15%) using EI mode. The molecular formula of compound C2 is (C$_{10}$H$_{10}$O$_4$). The IR (KBr, cm^{-1}) spectrum with the peaks at 3379 cm^{-1} (carboxylic acid O-H stretching), 2970 and 2900 cm^{-1} (CH-arom), 2742 cm^{-1} (CH-aliph.), 1716 cm^{-1} (carboxylic acid C=O stretching), 1265 cm^{-1} (carboxylic acid C-O stretching) and 1539; 1620 cm^{-1} (aromatic C=C).

The UV, IR, mass, 1H- and ^{13}C-NMR spectral data as well as co-chroma-tography with standard confirms the skeleton of ferulic acid.

Biological testing
Preparation of Suspensions

Isolated ferulic and coumaric acids were dissolved in DMSO and the volume was made up to 10 ml with distilled water to obtain a stock solution of 1 mg/ml concentration and stored at –20°C prior to use.

Table 1: Data of compound C1

Physically		40 mg powder, soluble in methanol
M.P.		212-214
R_f		0.66 in S1& 0.69 in S2
Color of spot in	Visible	-
	UV	Blue
	UV/NH$_3$	Yellowish green
	FeCl$_3$	Blue
UV λ $_{max}$ nm	MeOH	248,300,330
	NaOMe	240,265,370
^{1}HNMR (DMSO-d$_6$)δ ppm		δ: 12.47(s,1H,carboxylic proton), 9.31(s,board,1H, H-2), 7.64 (d,1H,J =15.8Hz, H-1'), 7.51(d,1H,J=7.7 Hz, H-3), 6.85 (d, H, J=2.2Hz, H-6), 6.83(dd,1H, J=2.2,7.7 Hz, H-5), 6.37 (d, H, J=15.8 Hz, H-2').
^{13}C-NMR(100 MHz, DMSO-d$_6$)		δ: 168.4(C-3'), 160.124(C-1), 146.07(C-6), 137.1(C-1'), 133.65(C-4), 126.2(C-3), 121.6(C-2'), 116.26(C-2), 115.6(C-5).

Table 2: Data of compound C2

Physically		50 mg crystals, soluble in methanol
M.P.		168-170
R_f		0.54 in S1& 0.66 in S2
Color of spot in	Visible	-
	UV	Blue
	UV/NH$_3$	violet
	FeCl$_3$	Blue
UV λ $_{max}$ nm	MeOH	219,230,400
	NaOMe	210,270,360
^{1}HNMR (DMSO-d$_6$)δ ppm		δ: 12.47 (1H,s, COOH), 7.51(1H, dd, J=8 and 2Hz, H-5), 7.49(1H, d, J=2.5Hz, H-3), 7.64(1H, d, J=15Hz, H-1'), 6.85(1H, d, J=9HZ, H-6), 6.27(1H,d,J=15 Hz, H-2'), 3.84(3H,s,H-4').
^{13}C-NMR(100MHz, DMSO-d$_6$)		δ: 179 (C-3'), 148.2 (C-1), 147.3 (C-6), 145.5 (C-1'), 136.26(C-4), 122.49 (C-3), 116.1 (C-2'), 115.5 (C-2), 103.54 (C-5) 55.8(C-4').

Further dilutions were made to obtain different concentrations ranging from 40 to 80 μg/ml with respective media and used for *in vitro* investigations.

Animals used

Adult albino rats (about 3-4 months old, 230-250 g) housed in temperature and humidity controlled room (24°C ± 2°C) with a 12-hr light/dark cycle and fed the standard laboratory diet and water ad-libitum were used. The guidelines issued by Animal Ethics Committee of October 6th University.

Hepatoprotective Effect of ferulic and coumaric acids in freshly isolated rat hepatocytes

The healthy rats were sacrificed by cervical decapitation and the healthy hepatocytes were performed using the collagenase perfusion method.[18] The obtained hepatocytes were suspended in Krebs-Henseleit buffer at a concentration of 5×10^6 cells/ml.

Paracetamol-induced in vitro hepatocytes injury

Paracetamol-induced hepatocytes injury was carried out. After an incubation of 24 h, the hepatocytes were exposed to paracetamol (5 mM) along with/without various concentrations (40, 50, 60, 70 and 80 μg/ml) of ferulic and/or coumaric acids or DMSO alone (as normal). After 60 min of paracetamol challenge, each sample was divided into two parts (0.5 ml each), one aliquot was used for the determination of TBARS level and the other one was centrifuged the clear supernatant was used for the determination of AST, ALT, ALP and LDH activities, while the residue was use for the estimation of GSH, SOD, CAT, GPx, GR, succinate dehydrogenase, protein thiol and total protein.

Biochemical investigation

Biochemical assays were carried out on isolated suspended hepatocytes as follows: activity of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) were carried out by Reitman and Frankel.,;[19] alkaline phosphatase (ALP) was carried out by Kind and King;[20] lactate dehydrogenase (LDH) was carried out by Buhl and Jackson,.[21] and TBARS was carried out by Uchiyama and Mihara,.[22] Levels of reduced glutathione (GSH), superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR), succinate dehydrogenase and protein thiols activities were carried out by Moron *et al*.,;[23] Marklund and Marklund,;[24] Sinha,;[25] Paglia and Valentine,;[26] Staal *et al*.,;[27] Jocelyn,;[28] Caplan and Greenawalt,[29] and Lowry *et al*.,[30] respectively.

Measurement of thiobarbaturic acid reactive substances (TBARS) level of hepatocytes

A thiobarbituric acid reactive substances assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, TBARS equivalents.[31] In brief, hepatocytes (0.5 ml) homogenized with 0.1 M sodium phosphate buffer (pH 7.4). One hundred microliters of homogenate was mixed with

2.5 mL reaction buffer (provided by the kit) and heated at 95°C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of TBARS equivalents.

Measurement of superoxide dismutase (SOD) activity of hepatocytes

The epinephrine method was used for superoxide dismutase (SOD) measurement. One mL of reaction mixture contained 50 mM sodium carbonate buffer (pH 10.0), 25 μL of 20 Mm epinephrine in 0.1 N HCl, and about 20 μg of protein in the enzyme sample. In the blank cuvette, the same amount of enzyme and buffer were taken, except epinephrine. Absorbance was recorded at 320 nm for 6 min. The activity was calculated using the difference between absorbance of standard and absorbance of enzyme and is expressed as units/mg protein.[24]

Measurement of Catalase (CAT) activity of hepatocytes

Catalase (CAT) activity of hepatocytes was determined at 25°C with spectrophotometer according to the previous study.[25] Diluted sample was added to 59 mmol/L H_2O_2 (dissolved in 50 mmol/L potassium phosphate buffer, pH 7.0) and CAT activity was measured at 240 nm for 3 min. One unit of CAT activity was defined as the mmol of H_2O_2 degraded/min/mg protein. The activity was expressed as U/mg protein in hepatocytes.

Measurement of Glutathione peroxidase (GPx) activity of hepatocytes

GPx activity of hepatocytes was determined with a commercial kit (Randox Laboratories, UK) according to the method by Paglia and Valentine,.[26] Twenty microliters of the diluted sample was added to 1 mL of mixed substrate (4 mmol/L GSH, 0.5 U/L GR and 0.34 mmol/L NADPH dissolved in 50 mmol/L phosphate buffer, pH 7.2, 4.3 mmol/L EDTA). Forty microliters of cumene hydroperoxide (diluted in deionized water) was added to the mixture and GPx activity was measured at 37°C on a Hitachi U-2000 Spectrophotometer at 340 nm for 3 min. The activity was expressed as mU/mg protein in hepatocytes.

Measurement of Glutathione reductase (GR) activity of hepatocytes

GR activity of hepatocytes was measured with a commercial kit (bio-diagnostic, USA) according to the method by Staal *et al.*,.[27] Two hundred microliters of the diluted sample was added to 400 μL of 2.4 mmol/L GSSG buffer (dissolved in 125 mmol/L potassium phosphate buffer, pH 7.5, 2.5 mmol/L EDTA). Four hundred microliters of 0.55 mmol/L NADPH (dissolved in deionized water) was added to the mixture and GR activity was measured at 340 nm for 5 min on a spectrophotometer. The activity was expressed as mU/mg protein in hepatocytes.

Measurement of succinate dehydrogenase activity of hepatocytes

Succinate dehydrogenase possesses dye reductase properties and is the only membrane bound enzyme in citric acid cycle. Phenazine methosulphate and 2, 6-dichlorophenol indophenol (DCIP) are electron acceptor dyes in the assay.[28] In this assay, the reaction mixture containing hepatocytes residue is mixed with 0.1 M potassium phosphate buffer (pH 7.4) and 0.1 M sodium cyanide and incubated at 37°C for 10 min. Sodium cyanide completely inhibits oxygen consumption in respiring cells and thus fairly prevents the loss of succinate by oxidation. The contents were split into two cuvettes followed by the addition of 2 mM phenazine methosulphate and 50 μM 2,6-dichlorophenolindophenol. Addition of 50 μL of 0.4 M succinate to the experimental cuvette marks the start of the reaction, whereas 50 μL of deionized water was added in the blank cuvette. Absorbance was recorded at 600 nm for 6 min.

Measurement of protein thiols level of hepatocytes

In this assay, hepatocytes residue was suspended in 14% perchloric acid, centrifuged at 4,500× *g* for 5 min, and the pellet was suspended in 7% perchloric acid and again centrifuged at 4,500× *g* for 5 min. To the pellet

were added, 10% Triton X-100, 0.2 M potassium phosphate buffer (pH 7.4). Subsequently, addition of 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB in the buffer was followed by measurement of absorbance at 412 nm after incubation for 5 min in the dark.[27]

Measurement of total reduced glutathione level of hepatocytes

About 60 μL of *O*-phosphoric acid was added to hepatocytes residue and centrifuged at 3000 rpm. The supernatant was then mixed with 0.1 M sodium phosphate buffer, 0.05 M EDTA (pH 8.0), and 100 μL *O*-phthaldehyde (1 mg/mL) and incubated at room temperature for 15 min to measure GSH levels. The absorbance was read at 410 nm against a reagent blank.[23]

STATISTICAL ANALYSIS

All data were expressed as mean of 6 replicates ± SD. All analyses utilized SPSS, 13.0 statistical package for Windows (SPSS, 13.0 software, Inc.,). A one-way analysis of variance(ANOVA) was employed for comparisons of means of the different groups. A p-value 0.05 was accepted as statistically significant.[32] Control positive (paracetamol group) was compared with control negative one. Ferulic and coumaric acids groups were compared with paracetamol treated group.

RESULTS

Structure elucidation of the isolated compounds

Buff powder and yellow crystals, obtained from C1 and C2 fractions. The structure of isolated compounds with melting point of 214 and 168°C is presented in Figure 1. ^{1}HNMR spectrum of ferulic acid displayed the characteristic signal for a methoxy group at δ 3.84 (s). Also, methoxy group signal was disappeared in ^{1}HNMR spectrum of coumaric acid (Table 1 and 2). The compounds spectrum also showed three aromatic proton at δ (6.85 & 6.8), (6.83 & 7.5) and (7.51 & 7.49) characteristics for the H-6, H-5 and H-3 of aromatic part of coumaric and ferulic acids, respectively. Also, The presence of further two protons doublets with J=15 Hz at δ (6.37 & 7.64) and (6.27 & 7.64) indicated the presence of H-2'and H-1' in the side chain of compounds, respectively.

The ^{13}CNMR spectrum showed the presence of specific signals (aromatic carbon, aliphatic chain and methoxy group) in agreement with the proposed structure of ferulic acid (4-hydroxy- 3-methoxycinnamic acid). Also, the ^{13}CNMR spectrum showed the absence of OCH_3 signal in agreement with the proposed structure of coumaric acid (4-hydroxy-cinnamic acid) (Table 1 & 2).

The EI-MS showed a molecular ion peak at m/z 164 and 194 (M[+], base peak) in agreement with the proposed structure of the known phenolic compound, coumaric and ferulic acids, with $C_9H_8O_3$ and $C_{10}H_{10}O_4$ molecular formula, respectively.

IR: The IR spectrum with the peaks at 3390 & 3379 cm^{-1} (carboxylic acid O-H stretching), 1672 & 1716 cm^{-1} (carboxylic acid C=O stretching), 1250 & 1256 cm^{-1} (carboxylic acid C-O stretching) and (1520, 1600) & (1539, 1620) cm^{-1} (aromatic C=C) confirms the skeleton of coumaric and ferulic acids, respectively. As it can be concluded, the IR spectrum of isolated compound is completely in agreement with the proposed structure of coumaric and ferulic acid.

Based on the above data and available literature, this is the first report to isolate coumaric acid and ferulic acid from the leaves of *Fortunella japonica* Swingle.

Biological activity

The effects of the isolated ferulic and coumaric acids on freshly isolated rat hepatocytes intoxicated with paracetamol are recorded in Table 3. A significant decrease in the levels of protein thiols and total protein levels ($P<0.05$) and a significant elevation in the levels of thiobarbituric acid reactive substances (TBARS) ($P<0.05$) were observed in hepatocytes exposed to paracetamol when compared to normal group.

Table 3: Effects of different concentrations of ferulic and coumaric acids treatment on protein thiols and total protein level as well as thiobarbituric acid reactive substances (TBARS) formation induced by paracetamol using isolated suspended rat hepatocytes

Groups		Total protein (g/dl)	Protein thiols (nmoles/mg protein)	TBARS (nmol/mg protein)
Normal		1.54± 0.16	10.83 ± 2.05	1.14 ± 0.20
Paracetamol (5 mM)		0.63 ± 0.04*	4.51 ± 0.60*	2.74 ± 0.13*
Ferulic acid	40 µg/ml	0.96 ± 0.07*	8.33 ± 1.37*	1.98 ± 0.24*
	50 µg/ml	1.34 ± 0.22*	8.90 ± 0.93*	1.60 ± 0.22*
	60 µg/ml	1.44 ± 0.17*	9.75 ± 1.15*	1.25 ± 0.16*
	70 µg/ml	1.58 ± 0.21*	10.45 ± 2.64*	1.17 ± 0.13*
	80 µg/ml	1.55 ± 0.15*	11.60 ± 1.80*	0.97 ± 0.08*
Coumaric acid	40 µg/ml	0.80 ± 0.09*	6.53 ± 0.87*	2.10 ± 0.26*
	50 µg/ml	0.96 ± 0.04*	6.88 ± 1.05*	1.90 ± 0.19*
	60 µg/ml	1.10 ± 0.22*	8.60 ± 1.33*	1.86 ± 0.11*
	70 µg/ml	1.34 ± 0.14*	9.50 ± 2.08*	1.53 ± 0.26*
	80 µg/ml	1.30 ± 0.11*	9.68 ± 1.48*	1.40 ± 0.30*

Values are given as mean ± SD for six replicates. Values are statistically significant at *$P<0.05$. Paracetamol group was compared with normal control. Ferulic and coumaric acids groups were compared with paracetamol group.

Table 4: Effects of different concentrations of ferulic and coumaric acids treatment on alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activity of paracetamol intoxicated freshly isolated rat hepatocytes

Groups		AST (U/L)	ALT (U/L)	ALP (U/L)
Normal		15.60 ± 2.15	17.38 ± 1.55	42.67 ± 3.85
Paracetamol (5 mM)		57.20 ± 4.08*	66.25 ± 4.86*	115.40 ± 11.27*
Ferulic acid	40 µg/ml	42.66 ± 5.37*	53.90 ± 5.38*	92.15 ± 9.80*
	50 µg/ml	36.14 ± 3.48*	39.26 ± 3.28*	80.48 ± 4.72*
	60 µg/ml	24.70 ± 2.15*	18.95 ± 2.36*	67.25 ± 5.06*
	70 µg/ml	18.43 ± 3.00*	15.27 ± 1.68*	42.73 ± 3.82*
	80 µg/ml	13.20 ± 2.30*	15.00 ± 2.09*	40.50 ± 2.96*
Coumaric acid	40 µg/ml	50.28 ± 4.37*	57.00 ± 4.21*	105.00 ± 9.60
	50 µg/ml	46.55 ± 3.81*	48.50 ± 4.10*	98.08 ± 6.38*
	60 µg/ml	33.00 ± 2.74*	39.64 ± 3.25*	76.30 ± 4.08*
	70 µg/ml	20.94 ± 2.50*	26.18 ± 3.05*	52.48 ± 5.11*
	80 µg/ml	18.00 ± 2.16*	19.05 ± 2.48*	45.11 ± 4.63*

Values are given as mean ± SD for six replicates. Values are statistically significant at *$P<0.05$. Paracetamol group was compared with normal control. Ferulic and coumaric acids groups were compared with paracetamol group.

Tables 4-6 showed that the paracetamol (5 mM) produced a marked increase in the leakage of ALT, AST, ALP and LDH within 60 min. of incubation period. Also, elevation in leaked of ALT, AST, ALP and LDH to 3 folds as compared to the respective negative control (p<0.05). Pre-incubation of the hepatocytes with ferulic and coumaric acids in combination (40, 50, 60, 70 and 80 µg) offered a significant protection against paracetamol-induced ALT, AST, ALP and LDH leakage as early as 60 min. as compared to the control positive group (p<0.05). The most effective dose for ALT, AST, ALP and LDH is 80 µg.

The results in of the present study illustrated that the paracetamol (5 mM) produced a marked depletion of SDH, SOD, CAT, GPx, GR and GSH within 60 min of incubation period as compared to the respective negative control group (p<0.01). Pre-incubation of the hepatocytes

with ferulic and coumaric acids showed a significant restoration of SDH, SOD, CAT, GPx, GR and GSH toward the normal (P<0.05, when compared with paracetamol treated group) and is dose dependent.

DISCUSSION

The isolated compounds were elucidated by, ^{1}HNMR, ^{13}CNMR, IR and MS as well as comparison of the data with those reported in the literature.[33]

Paracetamol is a commonly used, mild analgesic drug. It is considered safe in normal dosage, ingestion of large quantities of paracetamol can result in hepatic necrosis and acute renal failure in man [33]. This toxicity has been attributed to the formation of a highly reactive metabolic species, the N-acetyl-p-benzoquinone imine.[34]

Table 5: Effects of different concentrations of ferulic and coumaric acids treatment on superoxide dismutase (SOD), succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) activity as well as level of reduced glutathione (GSH) of paracetamol intoxicated freshly isolated rat hepatocytes

Groups		SOD (Units/min/mg protein)	SDH (nmoles DCIP red /min/mg protein)	LDH (U/L)	GSH (nmol/mg protein)
Normal		9.75 ± 1.07	42.25 ± 3.65	136.40 ± 10.75	3.94 ± 0.90
Paracetamol (5 mM)		3.40 ± 0.65*	13.68 ± 2.84*	310.72 ± 15.40*	1.65 ± 0.74*
Ferulic acid	40 μg/ml	6.83 ± 1.14*	20.96 ± 3.11*	259.80 ± 17.53*	2.51 ± 0.99*
	50 μg/ml	7.10 ± 2.16*	28.10 ± 4.07*	223.17 ± 15.84*	2.68 ± 0.63*
	60 μg/ml	8.45 ± 1.84*	36.50 ± 4.20*	194.50 ± 11.25*	2.74 ± 0.84*
	70 μg/ml	9.68 ± 1.25*	42.17 ± 4.84*	136.84 ± 13.48*	3.84 ± 0.56*
	80 μg/ml	9.95 ± 1.86*	44.00 ± 5.00*	132.36 ± 10.90*	3.86 ± 0.78*
Coumaric acid	40 μg/ml	5.44 ± 1.60*	15.29 ± 2.75	289.30 ± 14.27	2.35 ± 0.65
	50 μg/ml	6.80 ± 1.35*	19.58 ± 1.66*	265.00 ± 11.00*	2.70 ± 0.84*
	60 μg/ml	7.14 ± 1.72*	24.96 ± 3.48*	210.46 ± 17.52*	3.16 ± 0.77*
	70 μg/ml	8.66 ± 1.40*	35.60 ± 3.70*	175.19 ± 9.85*	3.64 ± 0.64*
	80 μg/ml	9.50 ± 1.81*	38.14 ± 4.08*	152.88 ± 12.65*	3.71 ± 0.93*

Values are given as mean ± SD for six replicates. Values are statistically significant at *$P<0.05$. Paracetamol group was compared with normal control. Ferulic and coumaric acids groups were compared with paracetamol group.

Table 6: Effects of different concentrations of ferulic and coumaric acids treatment on catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity of paracetamol intoxicated freshly isolated rat hepatocytes

Groups		CAT (U/mg protein)	GPx (mU/mg protein)x10^3	GR (mU/mg protein)
Normal		321.64 ± 10.87	3.73 ± 0.46	72.38 ± 4.50
Paracetamol (5 mM)		184.50 ± 8.64*	1.48 ± 0.35*	29.44 ± 3.71*
Ferulic acid	40 μg/ml	220.08 ± 9.04*	1.84 ± 0.26*	42.11 ± 3.96*
	50 μg/ml	264.36 ± 15.38*	2.93 ± 0.45*	54.86 ± 5.24*
	60 μg/ml	294.60 ± 12.75*	3.18 ± 0.61*	60.24 ± 6.28*
	70 μg/ml	310.85 ± 10.96*	3.70 ± 0.52*	73.60 ± 5.03*
	80 μg/ml	325.40 ± 16.48*	3.86 ± 0.41*	77.54 ± 4.25*
Coumaric acid	40 μg/ml	216.50 ± 17.33*	1.75 ± 0.28	38.50 ± 3.24
	50 μg/ml	257.36 ± 19.87*	1.94 ± 0.31*	46.17 ± 4.31*
	60 μg/ml	285.70 ± 12.65*	2.35 ± 0.25*	61.26 ± 3.88*
	70 μg/ml	305.26 ± 15.94*	2.96 ± 0.40*	68.00 ± 4.95*
	80 μg/ml	314.49 ± 21.00*	3.40 ± 0.53*	69.23 ± 5.18*

Values are given as mean ± SD for six replicates. Values are statistically significant at *$P<0.05$. Paracetamol group was compared with normal control. Ferulic and coumaric acids groups were compared with paracetamol group.

phenolics are now widely accepted as physiologic antioxidants that have a significant potential to protect against many degenerative diseases linked to free radical-related tissue damage.

The current study deal with the isolation of ferulic and coumaric acids from the leaves of *Fortunella japonica* Swingle and investigation of their structure antioxidant activity relationship in order to determine their possible roles in a paracetamol-induced liver toxicity.

These cells, when treated with the ferulic and coumaric acids showed a significant restoration of the altered biochemical parameters toward the normal when compared with paracetamol treated group and is dose dependent.

Lipids are known to react with oxidizing hydroxyl radical (·OH) to produce carbon-centered radical by: 1) hydrogen atom abstraction, and 2) addition to unsaturation. These lipid radicals (L·) react with oxygen to produce lipid peroxyl radical (LO2·), which initiates chain reaction causing lipid damage. The presence of coumaric and ferulic acids during paracetamol toxicity significantly inhibited the formation of these lipid oxidation products.

Coumaric and ferulic acids are known to react with oxidizing radicals induced by paracetamol and could serve as a strong free radical inhibitor or scavenger.[9,10]

Many attempts at explaining the structure activity relationships of some phenolic compounds have been reported in the literature.[35] It has been reported that the antioxidant activity of phenolic compounds may result from the neutralization of free radicals initiating oxidation processes, or from the termination of radical chain reactions, due to their hydrogen
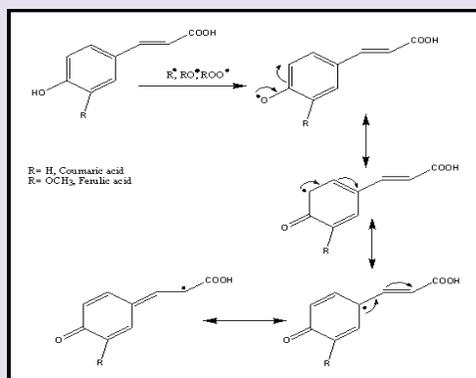
Figure 2: Proposal mechanism of coumaric and ferulic acids antioxidant activity

donating ability.[36] It is also known that the antioxidant activity of phenolic compounds is closely associated with their structures, such as substitutions on the aromatic ring and side chain structure.[37] It is also proposed that the higher antioxidant activity of compounds 2 and 3 is related to the presence of hydroxyl groups.[35] The structural requirements considered essential for effective radical scavenging by coumaric and ferulic acids is the presence of p-hydroxyl groups in aromatic ring and conjugated double bond. The presence of double bond between aromatic ring and carbonyl group in the isolated acids makes the electrons more delocalized to form quinone structure which possesses electron donating properties and is a radical target[37] (Figure 2).

These positive results suggest that coumaric and ferulic acids are a hepatoprotective agent against paracetamol induced hepatotoxicity. Therefore it may be considered as a potential molecules for alternative treatments of hepatocytes damage. It was further evaluated by a study done by Shanmugarajan et al.,[38] which confirmed the protective effects of ferulic acid against D-galactosamine; a hepatotoxin employed in studies involving liver disease, because it causes damage (necrosis) similar to the injury resultant of viral hepatitis in humans.[39]

Ferulic acid also has a hepatoprotective effect against toxicity induced in vivo by carbon tetrachloride, as reported by Srinivasan et al.,.[40] Treatment with the acid significantly decreased the index of lipid peroxidation in the liver and significantly increased the activities of superoxide dismutase, catalase and glutathione peroxidase.[41]

Coumaric and ferulic acids also imparted protection to the antioxidant enzyme SOD, CAT, GPx and GR from paracetamol-induced oxidative damage, which has been measured in terms of enzyme activity. The hepatocytes protective action of isolated acids toward SOD, CAT, GPx and GR has been found to be significant, even at 40 μg/ml, and increased from 50 to 80 μg/ml. The presence of isolated phenolic acids also inhibited the paracetamol-induced loss of activity of the hepatocytes marker enzyme SDH significantly from 40 to 80 μg/ml. The protection exerted by coumaric and ferulic acids against paracetamol-induced loss of endogenous antioxidant GSH has been found even at 40 μg/ml, and increased from 50 to 80 μg/ml concentration range.

Isolation and structure elucidation of coumaric and ferulic acids from *Fortunella japonica* Swingle leaves as well as their structure antioxidant activity relationship has not been reported earlier to our knowledge, and this study is perhaps the first observation of its kind.

Treatment with coumaric and ferulic acids significantly increased the activities of SOD, CAT, GPx and GR in isolated hepatocytes. Phenolic acids are shown to preserve physiological integrity of the cells exposed to various stress. This can be attributed to the effective antioxidant property of coumaric and ferulic acids. Normally phenolic compounds act by scavenging free radicals and quenching the lipid peroxidative side chain. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability and forming aryloxyl radicals.[42] It has been proposed that hydroxyl and hydroperoxyl radicals initiate H^{+} abstraction from a free phenolic substrate to form phenoxyl radical that can rearrange to quinonemethide radical intermediate,[43] which is excreted via bile. Srinivasan et al.,[40] have also been reported phenolic acids as a natural protector against carbon tetrachloride induced toxicity.

CONCLUSION

In conclusion, the present study indicates that coumaric and ferulic acids isolated from the leaves of *Fortunella japonica* Swingle are an efficient antioxidant against paracetamol-induced hepatocytes toxicity in isolated hepatocytes model, even at 40 μg/ml. the isolated phenolic acids are able to protect major biochemical components of the cells (lipids and proteins) from stress (paracetamol)-induced oxidative damage, and almost complete protection of lipids and proteins has been observed from 40 to 80 μg/ml. Other features like, natural occurrence and dietary isolated acids makes it attractive and suitable candidate as an antioxidant both *in vitro* and *in vivo*. Further, the ongoing studies with cell system may reveal the extent of protection exerted by coumaric and ferulic acids *in vivo* systems.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATION USED

UV: Ultraviolet; **IR:** Infrared, **NMR:** Nuclear Magentic Resonance spectroscopy, **SOD:** Superoxide dismutase; **SDH:** Succinate dehydrogenase; **CAT:** Catalase; **GPx:** Glutathione peroxidase and **GR:** Glutathione reductase; **ALT:** Alanine transaminase; **AST:** Aspartate transaminase; **LDH:** Lactate dehydrogenase and **ALP:** Alkaline phosphatase.

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PICTORIAL ABSTRACT
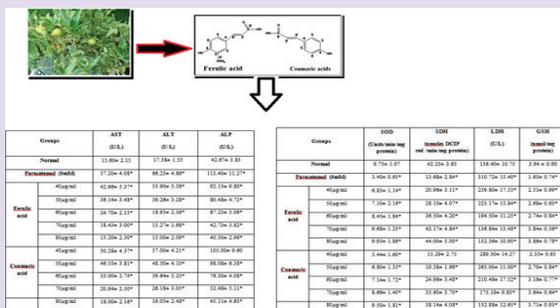
SUMMARY

- Ferulic and coumaric acids were isolated from *Fortunella japonica* Swingle leaves and their structural elucidation was supported using UV, IR, mass and Nuclear Magentic Resonance (NMR) spectroscopy.
- Ferulic and coumaric acids (40–80 μg/ml) show anti-oxidant and hepatoprotective activity against paracetamol-induced toxicity in freshly isolated rat hepatocytes model.
- Exposure of isolated hepatocytes to paracetmol (5mM) resulted in lipid hydroperoxides formation, depletion in protein thiols, superoxide dismutase (SOD), succinate dehydrogenase (SDH), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) levels as well as elevation of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP).
- Coumaric and ferulic acids have been found to efficiently inhibit paracetamol-induced biochemical alterations, namely oxidative stress biomarkers and protein oxidation.

ABOUT AUTHORS

Prof. Mohammed Abdalla Hussein: Ass. Prof. at department of Biochemistry, Faculty of Pharmacy, October 6 University, 6th of October City, Egypt. His research fields mainly focused on medical biochemistry, molecular biology, pharmaceutical importance of natural products, Drug design and delivery research. Based on this research and fellowship training he won several awards and honors, such as: October 6 University Incentive Award for Scientific Excellence in Biochemistry, December 2013 as well as Award of the best research article December 2014. He is serving as an editorial member of several reputed journals. He has published over 70 research articles in various peer reviewed journals.

Dr. Heba Abd-Elhady El-gizawy: Lecturer at Pharmacognosy department, Faculty of Pharmacy, October 6 University, Sixth of October City, Egypt. Her research fields mainly focused on the isolation of bioactive compounds from medicinal plants.