Secondary Metabolites of Ponderosa Lemon (*Citrus pyriformis*) and their Antioxidant, Anti-Inflammatory, and Cytotoxic Activities

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Column chromatography of the dichloromethane fraction from an aqueous methanolic extract of fruit peel of *Citrus pyriformis* Hassk. (Rutaceae) resulted in the isolation of seven compounds including one coumarin (citropten), two limonoids (limonin and deacetylnomilin), and four sterols (stigmasterol, ergosterol, sitosteryl-3-β-D-glucoside, and sitosteryl-6'-O-acyl-3-β-D-glucoside). From the ethyl acetate fraction naringin, hesperidin, and neohesperidin were isolated. The dichloromethane extract of the defatted seeds contained three additional compounds, nomilin, ichangin, and cholesterol. The isolated compounds were identified by MS (EI, CI, and ESI), ^{1}H, ^{13}C, and 2D-NMR spectral data. The limonoids were determined qualitatively by LC-ESI/MS resulting in the identification of 11 limonoid aglycones. The total methanolic extract of the peel and the petroleum ether, dichloromethane, and ethyl acetate fractions were screened for their antioxidant and anti-inflammatory activities. The ethyl acetate fraction exhibited a significant scavenging activity for DPPH˙ free radicals (IC_{50} = 132.3 μg/mL). The petroleum ether fraction inhibited 5-lipoxygenase with IC_{50} = 30.6 μg/mL indicating potential anti-inflammatory properties. Limonin has a potent cytotoxic effect against COS7 cells [IC_{50} = (35.0 ± 6.1) μM] compared with acteoside as a positive control [IC_{50} = (144.5 ± 10.96) μM].

Key words: Limonoids, Antioxidant, Anti-Inflammatory Properties

Introduction

Ponderosa lemon (*Citrus pyriformis* Hassk., Rutaceae) is a small tree, which is 3–6 m tall and native to southern Asia. The plant is primarily grown as an ornamental. It bears large fruits, which can weigh 1–2 kg. Ponderosa is considered as a natural hybrid between lemon and citron (*C. lemon* x *C. medica*) (Barrett and Rhodes, 1976).

The bioactive secondary metabolites from citrus plants have attracted great attention because of their diverse biological and pharmacological activities (Ladaniya, 2008; Manners, 2007; Tanaka *et al.*, 2008; Tripoli *et al.*, 2007). A survey of the literature revealed a few old reports on the identification of volatile oils (Shin'Ichi *et al.*, 2006; Yeh, 1955), hesperidin, neohesperidin (Horowitz and Gentili, 1960), citronin (Yamamoto and Oshima, 1931), and ergosterol (Nakamiya, 1931) in the rind and juice of *C. pyriformis* fruit peel. We have already studied the volatile constituents of the fruit peel of this plant (Hamdan *et al.*, 2010). Limonin isolated from this plant exhibited a significant inhibition of P-glycoprotein (P-gp) in human colon and leukemia cell lines (El-Readi *et al.*, 2010).

The aims of the present study were to characterize the chemical profiles of the fruit peel and seeds of *C. pyriformis* and to assess their antioxidant (DPPH˙ radicals scavenging activity) and anti-inflammatory (5-lipoxygenase inhibition) activities, respectively, as well as their cytotoxicity using Caco-2, COS7, and HeLa cell lines.

Abbreviations: FAB, fast-atom bombardment; FBS, fetal bovine serum; 5-LOX, 5-lipoxygenase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; NDGA, nordihydroguaiaretic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; ESI/MS, electrospray ionization/mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

Material and Methods

Plant material

The rind and seeds of *Citrus pyriformis* Hassk. (ponderosa lemon, *C. limon* Burm. f. var. *pyriformis*, *C. limon* 'ponderosa', *C. limon* 'American wonder') were separated from the ripe fruits collected in January 2005 from cultivated trees in the experimental station of the Faculty of Agriculture, Banha University, Banha, Egypt. The identity of plant was confirmed by Dr. B. M. Houlyel, Professor of Plant Pomology, Faculty of Agriculture, Banha University, Banha, Egypt.

Chemicals

Linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nordihydroguaiaretic acid (NDGA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Lipoxygenase from soybean (lyophilized powder) was supplied from Fluka (Buchs, Switzerland). Cell culture media, supplements, and dimethyl sulfoxide (DMSO) were purchased from Roth (Karlsruhe, Germany). Fetal bovine serum (FBS) was from Biochrome (Berlin, Germany), trypsin-EDTA and Hanks' balanced salt solution were bought from Gibco (Invitrogen, Karlsruhe, Germany). In addition, all solvents used for extraction, separation, and/or detection which were of analytical grade were obtained from Merck (Darmstadt, Germany).

Extraction and fractionation

The dried peel of *C. pyriformis* fruits (1.8 kg) was extracted with 80% aqueous methanol (3 × 8 L). The solvent was removed under reduced pressure to yield 508 g viscous extract. Part of the whole extract (430 g) was suspended in water (0.5 L) and subsequently subjected to fractionation using light petroleum (60–80 °C), dichloromethane, and ethyl acetate. The organic solvents were evaporated to yield 10 g, 15 g, and 31 g of final residue, respectively.

The dried seeds (100 g) were defatted with light petroleum and extracted with MeOH. The concentrated extract was suspended in H_2O and fractionated by extraction with CH_2Cl_2, and the solvent was concentrated to yield 3 g of a yellowish-white residue.

Isolation of compounds

Fruit peel

About 12 g of the dichloromethane fraction from the fruit peel were chromatographed on a silica gel column (150 × 2.5 cm, 250 g), packed in light petroleum. The polarity of the eluent was increased gradually using dichloromethane followed by methanol. Fractions of 250 mL each were collected, concentrated under vacuum, and monitored by TLC using pre-coated silica gel GF$_{254}$ plates (Merck) and a mixture of dichloromethane/acetone (9:1 or 8.5:1.5, v/v) and dichloromethane/methanol (9.8:0.2 or 9:1, v/v) as solvents for development. Spots were visualized by spraying with 10% (v/v) aqueous H_2SO_4 followed by heating at 105 °C for 5 min. By the aid of repeated crystallization and/or preparative layer chromatography of the column fractions compounds **1**–**7** were isolated. Their yields were 30, 10, 10, 6, 60, 40, and 75 mg, respectively.

The dried ethyl acetate fraction (10 g) was applied as dry mixed initial zone on the top of a silica gel column (150 × 5 cm, 300 g), packed in chloroform. The column was eluted with a gradient using a mixture of chloroform/methanol as mobile phase. Fractions were collected, concentrated, and monitored by TLC. The solvent for development was chloroform/aqueous methanol (6:4:0.5, v/v) and ethyl acetate/formic acid/acetic acid/water (10:1.1:1.1:2.6, v/v). Substances were detected under UV light and 10% (v/v) aqueous H_2SO_4. Three flavonoid glycosides, **8**–**10**, were isolated from the column eluates by crystallization and/or preparative TLC. The yields were 45, 40, and 1500 mg, respectively.

Seeds

The dried dichloromethane fraction (3 g) was chromatographed over a silica gel column (90 × 2.5 cm, 60 g). The column was eluted with cyclohexane, and the polarity of the eluent was increased gradually using ethyl acetate followed by methanol. Fractions of 100 mL each were collected, concentrated, and monitored by TLC using the solvent systems chloroform/methanol (9:1, 8:2, or 6:4, v/v) or cyclohexane/ethyl acetate (2:3, v/v). The spots were visualized under UV light, with 10% (v/v) aqueous H_2SO_4 and Ehrlich's spray reagents. Compounds **11**–**13** were isolated and their yields were 2, 5, and 1.5 mg, respectively.

Acid hydrolysis

The glycosidic compounds **8**–**10** isolated from the ethyl acetate fraction were individually sub-

jected to acid hydrolysis according to the procedure described by Harborne (1965). The organic layers were collected, concentrated, and crystallized to yield the corresponding aglycones. The aqueous phases were concentrated and spotted on paper chromatograms together with authentic sugars using ethyl acetate/formic acid/acetic acid/ water (10:1.1:1.1:2.6, v/v) for development.

Electrospray ionization/mass spectrometry (ESI/MS)

Electrospray ionization mass spectra of the dichloromethane fraction obtained from the defatted seeds were recorded on a VG QUATTRO II (ESI MS quadrupole) mass spectrometer (Waters, Manchester, UK). ESI/MS conditions were as follows: acquisition mode, ESI positive; mass scan range, *m/z* 350–500; capillary, 3.50 kV; HV lens, 0.50 kV; cone, 45 V; skimmer offset, 5 V; skimmer, 1.5 V; RF lens, 0.2 V; source temperature, 82 °C; pressure for analyser vacuum and gas cell, 1.8^{e-5} and 1.5^{e-4} mbar, respectively. Drying and nebulizing gas was nitrogen. Ten mg of dichloromethane fractions were dissolved in MeOH (1 mL) and 100 µL from these solutions were taken and diluted with 500 µL 2% (v/v) formic acid in acetonitrile/water (1:1, v/v). One hundred µL from each diluted solution were qualitatively analysed by direct introduction into the ESI positive ion mode.

Liquid chromatography coupled with ESI/MS

The chromatographic separation of the dichloromethane fraction of the seeds was carried out by HPLC using an RP C-18 LiChro CART (Merck) column (250 × 4 mm, 5 µm). The mobile phase consisted of solvent A [water/formic acid (99.5:0.5, v/v] and solvent B (acetonitrile). The elution was arranged as follows: 0–5 min, isocratic at 0% B; 5–110 min, gradient from 0–70% B; 110–115 min, gradient from 70–100% B; 115–140 min, isocratic at 100% B. The column was equilibrated with 100% solvent A for 10 min prior to each analysis. Flow rate was 1 mL/min, and only 10% of the total eluent were carried to MS detection. Injection volume was 100 µL. The analysis was performed under the following conditions: acquisition mode, ESI positive; nebulizer gas, N₂, 0.25 L/ min; capillary, 3.00 kV; HV lens, –0.50 kV; cone, –35 V; source temperature, 120 °C; RF lens, –0.2 V; skimmer, –1.5 V; mass scan range, *m/z* 200–800; interscan time, 0.1 s. MS data were acquired and

processed using MassLynx $V_{4.0}$ software. The limonoid content of the seeds was analysed by dissolving the dichloromethane extract (residue) in DMSO (10 mg/mL, w/v). Electron impact mass spectra were recorded on a Finnigan MAT 8200 (Bremen, Germany) instrument with electron energy of 70 eV. In case of FAB-mass spectrometry, 3-nitrobenzyl alcohol was used as a matrix.

NMR analysis

NMR spectra (^{1}H and ^{13}C) were recorded on VARIAN Mercury 300 and VARIAN 500 instruments (Darmstadt, Germany) at 300 and 500 MHz for ^{1}H measurements and 75 and 125 MHz for ^{13}C measurements, respectively. CD₃OD, DMSO-d_6, and CDCl₃ were used as solvents. Chemical shifts are determined in ppm with TMS as internal standard. APT, 2D-NMR, H-H COSY, and HMPQC experiments were applied to obtain reliable assignments. Experimental data were processed using MestRe-C software.

Antioxidant activity

The free radical scavenging activity of the crude methanolic extract and solvent fractions (petroleum ether, dichloromethane, and ethyl acetate) were tested separately. Stock solutions of the total extract and fractions were prepared by dissolving 10 mg in 1 mL methanol. Serial dilution was done from each stock solution, and 500 µL from each dilution were tested individually by adding to 500 µL of 0.2 mM DPPH; the final volume was brought to 1 mL. The mixtures were vigorously shaken and allowed to stand in the dark for 30 min at room temperature. The absorbance was measured by spectrophotometry (LKB Pharmacia Biochrom ULTROSPEC PLUS 4054 UV/VIS; Freiburg Germany) at 517 nm against a blank sample without DPPH (negative control). The antioxidant activity expressed as IC_{50} (µg/mL) was compared to standard antioxidants such as ascorbic acid (Ricci *et al.*, 2005). Inhibition of DPPH radicals was calculated using the equation (Dordevic *et al.*, 2007) $I\ (\%) = 100 \cdot (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound) and A_s is the absorbance of the test sample.

Inhibition of 5-lipoxygenase

Inhibition of soybean 5-lipoxygenase (5-LOX) by the total extract and different solvent fractions (petroleum ether, dichloromethane, and ethyl

acetate) was determined spectrophotometrically (Baylac and Racine, 2003) under the following conditions: to 970 μL of phosphate buffer (21.2 g K_3PO_4 in 1 L H_2O), pH 9.0, 10 μL of 1 mg/mL 5-LOX and 20 μL of 9 different concentrations of the test samples (16–250 μg/mL) were added; the mixture was incubated at room temperature for 10 min. The enzymatic reaction was initiated by adding 25 μL of 62.5 mM sodium linoleate, and the reaction kinetics were monitored at 234 nm every 10 s for 3 min using a LKB Pharmacia Biochrom spectrophotometer. The initial reaction rates were determined from the slope of the straight-line portion of the curve, and inhibition of the enzyme activity was calculated from triplicate experiments. NDGA was used as a positive reference compound with an IC_{50} value of (0.2 ± 0.1) μg/mL.

Cell culture

Caco-2, HeLa, and COS7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (glutamate I) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. To the HeLa cell medium, 10 mM non-essential amino acids were added; in case of Caco-2 cell medium, 1 mM sodium pyruvate and 10 mM non-essential amino acids were added. Cell lines were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay

Sensitivity of the cells to secondary metabolites was determined in triplicate using the MTT cell viability assay (Mosmann, 1983). Exponentially growing Caco-2 cells (2 · 10^4 cells/well), COS7, and HeLa cells (5 · 10^4 cells/mL) were seeded in a 96-well plate (Greiner Labortechnik, Frickenhausen, Germany) after trypsinization of a subconfluent culture flask. After incubation for 24 h the cells were incubated with fresh medium containing various concentrations of compounds at 37 °C for 24 h. The medium was removed and cells were incubated with fresh medium containing 0.5 mg/mL MTT from a 5 mg/mL stock solution in phosphate buffered saline (PBS). During incubation for further 4 h MTT was reduced by mitochondrial dehydrogenases of viable cells to a purple formazan product. The medium was discarded and formazan crystals dissolved in 100 μL DMSO. The plates were shaken 15 min at room temperature, and the absorbance was detected at 570 nm using a Tecan Ultra ELISA Plate Reader (Männedorf, Switzerland). Data are presented as IC_{50} values (μM) (mean ± SD).

Statistical analysis

All experiments were carried out at least three times. Continuous variables are presented as mean ± standard deviation (SD). The IC_{50} value was determined as the drug concentration which resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC_{50} values were calculated using a four-parameter logistic curve (SigmaPlot 11.0).

Results and Discussion

Identification of isolated compounds

Altogether 10 compounds were isolated and identified from the dichloromethane fraction of the aqueous methanolic extract of the fruit peel of *C. pyriformis* (Fig. 1). The dichloromethane extract of the defatted seeds revealed three compounds. The chemical structures of the isolated compounds were identified by interpretation of their spectral data including MS, 1D- and 2D-NMR experiments (APT, COSY, HSQC, HMBC, and NOESY) and comparison with literature data as well as authentic substances from our laboratory. The isolated compounds include citropten (limettin) (**1**) (Osborne, 1989), stigmasterol (**2**), ergosterol (**3**) (Goad and Akishisa, 1997), sitosteryl-6'-*O*-acyl-3-β-D-glucoside (**4**) (Goad and Akishisa, 1997; Guerriero *et al.*, 1991), limonin (**5**), deacetylnomilin (**6**) (Khalil *et al.*, 2003; Nakagawa *et al.*, 2001), sitosteryl-3-β-D-glucoside (**7**) (Goad and Akishisa, 1997; Guerriero *et al.*, 1991), naringin (**8**), hesperidin (**9**), neohesperidin (**10**) (Maltese *et al.*, 2009), cholesterol (**11**) (Goad and Akishisa, 1997), nomilin (**12**), and ichangin (**13**) (Khalil *et al.*, 2003; Nakagawa *et al.*, 2001). The isolated compounds are reported here for the first time as constituents of *C. pyriformis* except ergosterol (Nakamiya, 1931), hesperidin, and neohesperidin (Horowitz and Gentili, 1960), which had been previously reported from this plant.

ESI/MS

Analysis of the dichloromethane fraction of the defatted seeds by direct ESI/MS (positive mode) revealed peaks with the following molecular masses ([M+H]$^+$): 457, 471, 473, 488, 491,
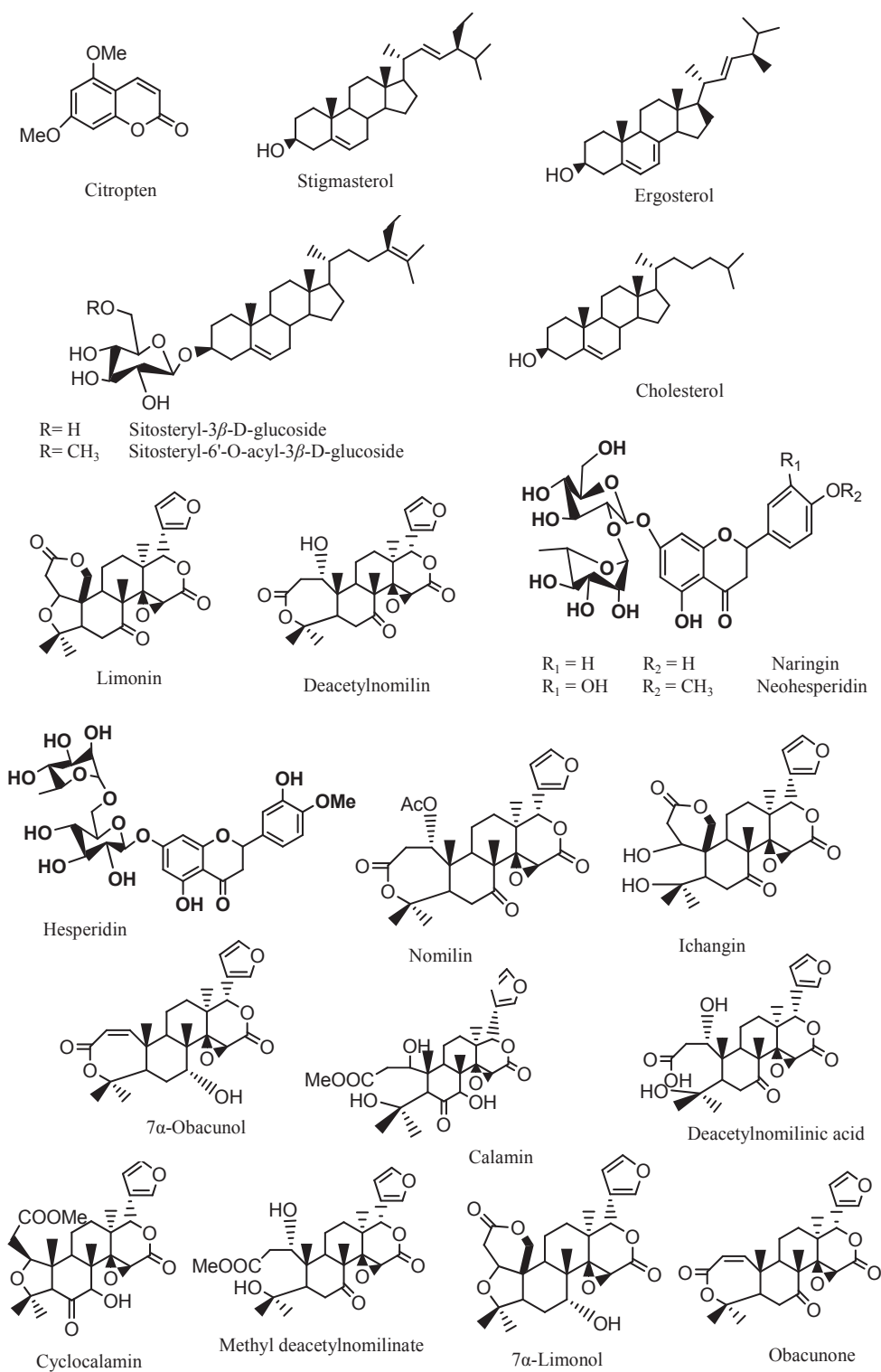
Fig. 1. Isolated and identified compounds from the peel and seeds of *C. pyriformis*.

503, and 515. These molecular ions correspond to the following compounds: 7α-obacunol, limonin, deacetylnomilin, ichangin, deacetylnomilinic acid, cyclocalamin, and nomilin, respectively. Limonin, deacetylnomilin, ichangin, and nomilin were identified by comparison of the data with those of authentic samples. Based on chromatographic and MS data, the other limonoids (7α-obacunol, deacetylnomilinic acid, and cyclocalamin) were tentatively identified.

LC-ESI/MS

The combination of HPLC and ESI/MS is a simple, sensitive, and selective method for separation and identification of complex limonoid mixtures. Relative retention times and molecular masses provide sufficient information for unequivocal identification of most of the limonoids, especially those, which are present as trace components or of geometrical isomers (Liang *et al.*, 2005; Manners, 2007; Manners *et al.*, 2003; Manners and Hasegawa, 1999; Tian *et al.*, 2000). The limonoid profiles of the dichloromethane extract of defatted seeds were analysed by LC-ESI/MS (Fig. 2). Identified secondary metabolites include methyl deacetylnomilinate, 7α-limonol, deacetylnomilinic acid, ichangin, deacetylnomilin, limonin, nomilin, obacunone, and calamin. The amounts of other limonoids were too small to carry out separation, full characterization, and complete assignments. Thus, a few compounds were tentatively identified by comparison of their chromatographic (relative

Table I. DPPH· scavenging activity of total extract and fractions from *C. pyriformis*.

Sample	IC_{50} [μg/mL]
Total extract	625.9 ± 0.8
Petroleum ether fraction	445.8 ± 0.7
Dichloromethane fraction	149.9 ± 1.2
Ethyl acetate fraction	132.3 ± 0.6
Ascorbic acid	16.3 ± 0.2

Data are means ± SD from three independent experiments ($n = 3$).

retention time) and MS data with those reported in the literature (Manners and Hasegawa, 1999).

Antioxidant activity

The IC_{50} values as a measure for the antioxidant properties of the total extract and different fractions are given in Table I. The ethyl acetate fraction exhibited the highest DPPH· free radical scavenging activity (IC_{50} = 132.3 μg/mL), while the total methanolic extract was less active (IC_{50} = 625.9 μg/mL).

Inhibition of 5-lipoxygenase

Inhibition of 5-LOX can be an indication of antiallergic and anti-inflammatory properties of secondary metabolites. NDGA was used as positive reference compound (Abad *et al.*, 1995; Safayhi *et al.*, 1992). The IC_{50} values of the total extract and different fractions are summarized in Table II. The petroleum ether fraction inhibited 5-LOX
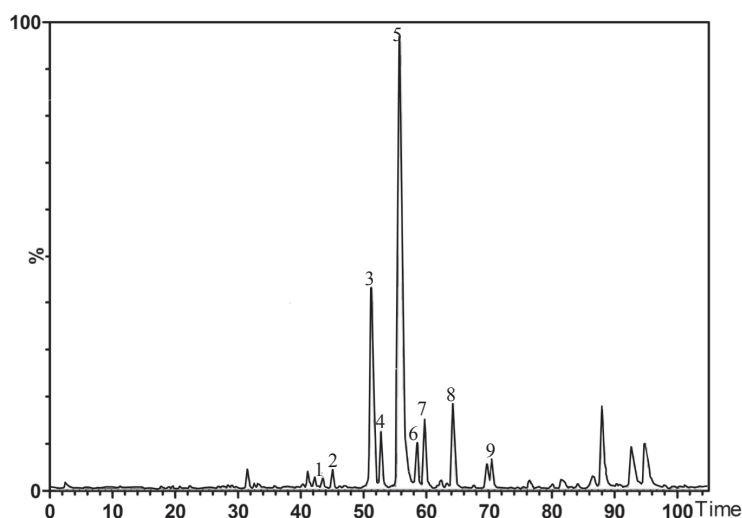

Fig. 2. LC-ESI/MS chromatogram of the dichloromethane fraction from the defatted seeds of *C. pyriformis*. The identified compounds were: 1, methyl deacetylnomilinate; 2, 7α-limonol; 3, deacetylnomilinic acid; 4, ichangin; 5, deacetylnomilin; 6, limonin; 7, calamin; 8, nomilin; 9, obacunone. The undefined peaks are of unknown compounds.
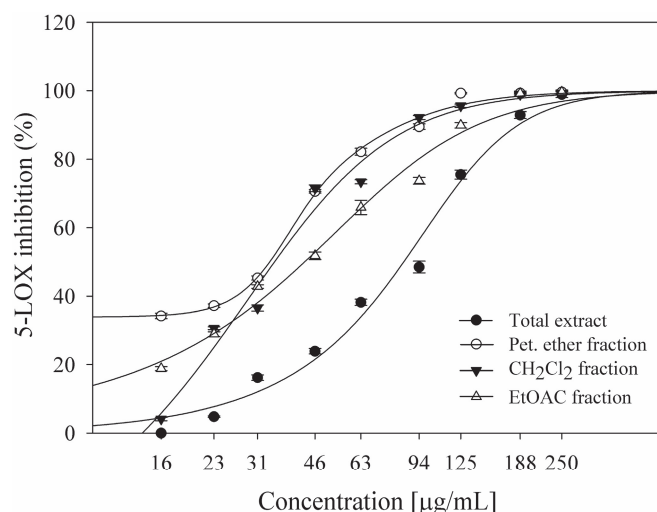
Fig. 3. Dose-response curves of 5-lipoxygenase inhibition by fractions of *C. pyriformis*.

Table II. Inhibition of 5-lipoxygenase by fractions of *C. pyriformis* in %.

Concentration [μg/mL]	Total extract	Petroleum ether fraction	Dichloromethane fraction	Ethyl acetate fraction
16	0.00	34.2 ± 0.8	4.1 ± 0.5	18.8 ± 0.6
23	4.8 ± 0.2	37.2 ± 0.5	30.6 ± 0.4	28.9 ± 0.7
31	16.2 ± 0.8	45.3 ± 0.6	36.5 ± 0.9	42.8 ± 0.5
46	23.9 ± 0.8	70.6 ± 0.5	71.5 ± 1	51.8 ± 1.1
63	38.2 ± 0.9	82.2 ± 1	73.4 ± 0.6	65.9 ± 2.1
94	48.5 ± 1.7	89.5 ± 0.8	92.2 ± 0.6	73.7 ± 1
125	75.5 ± 1.3	99.3 ± 0.2	95.6 ± 0.5	89.9 ± 0.8
188	92.9 ± 1	99.3 ± 0.3	98.9 ± 0.4	99.2 ± 0.7
250	98.9 ± 0.9	99.6 ± 0.6	99.3 ± 0.4	99.6 ± 0.4
IC_{50}[a]	96.3 ± 2	30.6 ± 0.5	37.3 ± 0.9	42 ± 1.1

Data are means ± SD from three independent experiments (n = 3).
[a] IC_{50} values in μg/mL.

Table III. Cytotoxicity of compounds isolated from *C. pyriformis* against Caco-2, HeLa, and COS7 cells; data represent IC_{50} values in μM.

Sample	Caco-2	HeLa	COS7
Limonin	519.8 ± 12.5	132.1 ± 8.2	35.0 ± 6.1
Deacetylnomilin	477.5 ± 22.2	113.2 ± 7.1	85.1 ± 7.4
Sitosteryl-3-β-D-glucoside	387.1 ± 10.4	121.2 ± 8.9	58.9 ± 4.4
Acteoside	294.2 ± 20.1	78.5 ± 3.6	144.5 ± 10.96
Neohesperidin	174.1 ± 19.2	123.5 ± 9.5	51.6 ± 4.9
Hesperidin	194.9 ± 43.9	106.2 ± 12.5	51.5 ± 3.9
Sterol mixture	337.8 ± 9.3	114.2 ± 12.3	30.3 ± 0.7

Data are means ± SD from three independent experiments (n = 3).

with an IC_{50} value of 30.6 μg/mL while the total methanolic extract was less active (IC_{50} = 96.3 μg/mL). Fig. 3 shows the dose-response curves of the total extract and different fractions of *C. pyriformis*.

Cytotoxicity

Limonin and deacetylnomilin inhibited the growth of HeLa cells with an IC_{50} value of 132.1 μM and 113.2 μM, respectively. The activity of both limonoids is probably related to their epoxy groups which are very reactive and can bind to nucleophilic groups (NH_2, NH, and SH) of amino acid residues in proteins (Wink, 2008). In case of COS7 cells, the IC_{50} value of deacetylnomilin was more than twice as high that of limonin (IC_{50} = 85.1 μM and 35.0 μM, respectively). This means that limonin is more cytotoxic for COS7 cells than deacetylnomilin although both compounds have an epoxy group. This is probably due to the free hydroxy group in deacetylnomilin, which may form hydrogen bonds with water molecules and reduces free diffusion across the cell membrane while limonin is more lipophilic and can enter the cell more easily. In case of the Caco-2 cell line, limonin and deacetylnomilin did not or only minimally inhibit cell growth [IC_{50} = (519.8 ± 12.5) μM and (477.5 ± 22.2) μM], whereas their toxicity was 5 times higher in HeLa and COS7 cells (Table III). The low sensitivity of Caco-2 cells may be due high expression of P-gp, an ABC transporter, which pumps out lipophilic compounds that have entered the cells by free diffusion (El-Readi et al., 2010).

Sitosteryl-3-β-D-glucoside was also studied in the same cell lines; in case of HeLa and COS7 cells its IC$_{50}$ values were 121.2 and 58.9 μM, respectively. However, the sterol glucoside hardly inhibited the growth in Caco-2 cells (Table III). All tested samples showed high IC$_{50}$ values in the chemotherapy-resistant Caco-2 cell line in comparison to the sensitive HeLa and COS7 cells, which have a low endogenous ABCG2 and P-gp expression (El-Readi *et al.*, 2010).

The isolation and detection of bioactive secondary metabolites (limonoids, flavonoids, and sterols) in extracts of *C. pyriformis* as well as their promising biological properties can explain the use of this plant in traditional medicine. It may be a natural source of lead substances for new therapeutic applications.

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