Food Chemistry 127 (2011) 394–403

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical composition and biological activity of *Citrus jambhiri* Lush

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ARTICLE INFO

Article history:
Received 17 February 2010
Received in revised form 28 October 2010
Accepted 29 December 2010
Available online 8 January 2011

Keywords:
Citrus jambhiri
Polymethoxyflavones
Flavonoid glycosides
Limonoids
NMR
LC–ESI/MS
Antioxidant
Anti-inflammatory
Cytotoxicity

ABSTRACT

The fresh peel of *Citrus jambhiri* was extracted with aqueous methanol and the residue was fractionated using light petroleum, chloroform and ethyl acetate. The constituents of the extracts were separated by column chromatography employing solvents of different polarity. The chemical structure of the isolated compounds was then identified by MS and NMR. Column chromatography of the petroleum fraction resulted in the isolation of nobiletin, 5-*O*-demethylnobiletin, tangeretin, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and a mixture of *β*-sitosterol and stigmasterol. The chloroform fraction afforded 6-demethoxynobiletin, 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone, limonin and nomilin. The flavonoid glycosides naringin, hesperidin and neohesperidin were isolated from the ethyl acetate fraction. The chemical structure of the isolated compounds was established by MS and NMR (APT, COSY, HSQC, HMBC, and NOESY). LC–ESI-MS analysis of the ethyl acetate fraction afforded eight flavonoid glycosides, while the dichloromethane fraction of the defatted seeds contained seven limonoid aglycones. The chloroform fraction exerted the strongest DPPH* free radical scavenging activity in comparison to other fractions. The petroleum fraction showed a significant inhibition of lipoxygenase indicating an anti-inflammatory action (IC_{50} 29 ± 1 μg/mL). Some of the isolated polymethoxyflavones exhibited strong cytotoxicity against COS7, HeLa and Caco-2 cell lines.

1. Introduction

Citrus fruits (Rutaceae) possess high amounts of bioactive compounds which can influence human health, as e.g. vitamin C, carotenoids (β-carotene), flavonoids, limonoids, essential oils, coumarins, acridone alkaloids, high quality soluble fibre, minerals, vitamin-B complex and related nutrients such as thiamine, riboflavin, nicotinic acid/niacin, pantothenic acid, pyridoxine, folic acid, biotin, choline, and inositol (Ladaniya, 2008); health promoting effects include antioxidant, cardioprotective, anticarcinogenic, anti-allergic, antiplatelet, antiviral, antibacterial and antifungal activities (Manners, 2007; Miller, Porter, Binnie, Guo, & Hasegawa, 2004; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). Lemon juice has had a long tradition in folk medicine as being used for treating the common cold, and as a diuretic, antiscorbutic, astringent and fever-reducing remedy.

Flavonoids, in particular polymethoxyflavones, flavanone glycosides and limonoids, are naturally abundant secondary metabolite compounds in *Citrus* species, and since these substances bear ecological, biological and chemotaxonomic importance in this genus (Ladaniya, 2008; Manners, 2007; Tripoli et al., 2007), they have attracted considerable interest.

A review of the literature indicates that only few studies have been conducted on the fruit peel constituents. Coumarins have been isolated and detected from different organs of *Citrus jambhiri*, such as 6,7-dimethoxycoumarin (Sulistyowati, Keane, & Anderson, 1990), aurapten (Ogawa et al., 2000), bergapten and psoralin (McCloud, Berenbaum, & Tuveson, 1992). The flavonoid glycosides hesperidin and neohesperidin have been detected in the fruit juice using high-performance liquid chromatography (HPLC) (Arriaga & Rumbero, 1990). Also, the polymethoxyflavones tangeretin, 5-*O*-demethyltangeretin and 5-hydroxy-6,7,8,4′-tetramethoxyflavone have been reported from this species (Chaliha, Sastry, & Rao, 1965; Tatum & Berry, 1972).

The aim of this study has been to investigate the chemical composition, as well as the antioxidant, anti-inflammatory and *in vitro* cytotoxic activities, of the total methanolic extract, light petroleum, chloroform and ethyl acetate fractions and of some secondary metabolites isolated from *C. jambhiri* in order to evaluate its pharmacological potential.

2. Materials and methods

2.1. Plant material

The fresh fruit rind and seeds of rough lemon (*C. jambhiri* Lush.), Rutaceae, were separated from the ripe fruits which were collected

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doi:10.1016/j.foodchem.2010.12.129

from the Research Station of the Faculty of Agriculture, Benha University, Egypt in March 2004. The identity of the plants was confirmed by Prof. Dr. B. M. Houlyel, Dept. of Pomology, Faculty of Agriculture, Benha University, Egypt.

2.2. Chemicals

2,2 Diphenyl-1-picryl-hydrazil radical (DPPH), linoleic acid sodium salt, nordihydroguaiaretic acid (NDGA), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and ascorbic acid were purchased from Sigma–Aldrich® (Taufkirchen, Germany). Lipoxydase enzyme from soybean (lyophilised powder) was supplied from Fluka (Buchs, Switzerland). Cell culture DMEM-glutamate I media, dimethyl sulphoxide (DMSO) were purchased from Roth® (Karlsruhe, Germany). Foetal bovine serum (FBS) from Biochrome® (Berlin, Germany), trypsin–EDTA, penicillin, streptomycin, sodium pyruvate, non-essential amino acids and Hanks' balanced salt solution were bought from Gibco® (Invitrogen; Karlsruhe, Germany). Authentic compounds; rutin, eriocitrin, neoeriocitrin, diosmetin 6-C-glucoside, narirutin, naringin, hesperidin, neohesperidin, limonin, nomilin, and palmitic acid were obtained from university of Heidelberg, IPMB. In addition, methanol, dichloromethane, chloroform, petroleum ether, benzene, ethylacetate, formic acid, acetonitrile and other solvents used for extraction, separation and/or detection, which were of analytical grade, were obtained from Merck (Germany).

2.3. Extraction procedure

Fresh peel of *C. jambhiri* Lush. (5 kg) was exhaustively extracted with 80% aqueous methanol (3 × 10 L). The methanolic extract was filtered and then concentrated under vacuum to yield 1750 g of a viscous residue. The residue was suspended in water and partitioned against light petroleum (b.p. 60–80 °C), chloroform and ethyl acetate successively. The organic solvents were evaporated under vacuum using rotary evaporator at lower temperature to yield 20, 15 and 20 g of final residue, respectively.

Dried seeds (290 g) were cleaned, further dried, powdered, defatted with light petroleum and extracted with methanol (MeOH). The methanolic extract evaporated under vacuum using rotary evaporator at 55 °C to give 24 g residue which diluted with water (H_2O), extracted with dichloromethane (CH_2Cl_2) and the solvent was evaporated under vacuum to yield 9 g of residue.

2.4. Isolation of the compounds

2.4.1. Compounds 1–7

The light petroleum fraction (8 g) redissolved in dichloromethane and the solution was mixed with 10 g silica for column. The dry mixed initial zone was chromatographed on a silica gel column (150 × 2.5 cm, 200 g) at room temperature. The column was eluted with a gradient using a mixture of benzene–chloroform–ethyl acetate as mobile phase. Fractions 250 mL were collected and monitored by thin layer chromatography (TLC) using pre-coated silica gel GF_{254} (Merck) and a mixture of chloroform–ethyl acetate (8:2, v/v) for TLC development. Spots were visualised by spraying with H_2SO_4 10% (v/v) in water followed by heating at 105 °C for 5 min.

Fractions 23–36 eluted at a benzene/chloroform (C_6H_6-$CHCl_3$), (8:2, v/v) were collected, evaporated and after crystallisation in a chloroform/methanol mixture compound **1** was obtained as a white amorphous powder (12 mg) with retention factor (Rf) 0.77. Additionally, fractions 37–50 eluted at a C6H6-$CHCl_3$, (8:2, v/v) were pooled and compound **2** (15 mg) was collected as white needle-shaped crystals from chloroform/methanol with Rf 0.74. Moreover, fractions 63–69 eluted at a C_6H_6-$CHCl_3$, (1:9, v/v) were collected and chromatographed again on the prep-TLC which was developed using the solvent system dichloromethane-acetone, (9:1, v/v) and eluted to afford compound **3** (8 mg) as a yellow amorphous powder with Rf an 0.66.

The column fractions 70–85 eluted at a benzene-$CHCl_3$, (1:9, v/v) were pooled and crystallised in chloroform/acetone producing 100 mg of compound **4** as yellowish-white needle-shaped crystals with Rf 0.54. In addition, fractions 86–101 eluted with 100% chloroform ($CHCl_3$), gave 15 mg of compound **5** as dark yellow needle-shaped crystals (from hot chloroform/methanol) with Rf 0.35. Fractions 102–106 eluted at a chloroform/ethylacetate ($CHCl_3$-EtOAc), (9.5:0.5, v/v) were pooled and crystallised in hot chloroform/methanol to afford compound **6** (40 mg) as dark yellow needle-shaped crystals with Rf 0.28. Fractions 107–115 eluted at a $CHCl_3$-EtOAc, (9:1, v/v) were collected and crystallised in chloroform/methanol to yield compound **7** as yellow granules (25 mg) with Rf 0.25.

2.4.2. Compounds 8–11

The dry mixed initial zone was prepared from 8 g of chloroform fraction and was chromatographed on a silica gel column (150 × 2.5 cm, 200 g) at room temperature. The column was eluted with a gradient using mixture of benzene–chloroform–ethyl acetate as mobile phase. Fractions of 250 mL each were collected, concentrated under vacuum and monitored by TLC using a mixture of chloroform–ethyl acetate, (8:2, v/v) as a system for TLC development. Spots were detected by UV after spraying with H_2SO_4 10% (v/v) in water and/or Ehrlich's reagents. Column fractions 45–50 eluted at a C_6H_6-$CHCl_3$, (1:9, v/v) were collected and 15 mg of compound **8** was crystallised as yellowish-white needles from chloroform–methanol with Rf 0.28. Fractions 66–70 eluted at a $CHCl_3$-EtOAc, (9.7:0.3, v/v) were concentrated and crystallised in chloroform/methanol yielding 10 mg of compound **9** as white needle-shaped crystals with Rf 0.21. Column fractions 71–80 eluted at a $CHCl_3$-EtOAc, (9.5:0.5, v/v) were crystallised in chloroform/methanol producing 650 mg of compound **10** as a white amorphous powder with Rf 0.16. The collected fractions 81–91 eluted at a $CHCl_3$-EtOAc (9:1, v/v) afforded 12 mg of compound **11** as yellow granules with Rf 0.11.

2.4.3. Compounds 12–14

Dry mixed initial zone was done by dissolving 8 g of ethyl acetate fraction in methanol (10 mL), load the solution over silica for column, triturate the mixture till drying and the dried zone was chromatographed on a silica gel column (150 × 4 cm, 300 g) at room temperature. The column was eluted with a gradient using mixture of chloroform–methanol as mobile phase. Fractions of 250 mL each were collected, concentrated under vacuum and monitored by TLC using ethyl acetate-formic acid-acetic acid–water, (10:1.1:1.1:2.6, v/v) as developing system. The TLC was visualised under UV and H_2SO_4 10% (v/v) in water spray reagent. Fractions 31–50 eluted at a $CHCl_3$-MeOH, (9:1, v/v) were crystallised in methanol to give 10 mg of compound **12** as buff granules with Rf 0.77. Crystallisation of column fractions 51–65 eluted at a $CHCl_3$-MeOH, (8:2, v/v) gave 800 mg of compound **13** as white granules using hot methanol with Rf 0.72. Column fractions 66–75 eluted at a $CHCl_3$-MeOH (8:2, v/v) were collected, concentrated and rechromatographed on a preparative TLC which was developed using chloroform–methanol–water, (6:4:0.5, v/v) and eluted to afford compound **14** (15 mg) as white granules which obtained by crystallisation using hot methanol with Rf 0.67.

2.5. Acid hydrolysis

Compounds **12–14** which had been isolated from the ethyl acetate fraction were subjected to acid hydrolysis by dissolving about 5 mg of each compound in 5 mL methanol and refluxing with

20 mL aqueous H_2SO_4 (50%) for 6 h. The reaction mixtures were extracted twice with chloroform (50 mL). The organic layers were collected, concentrated and crystallised to yield the corresponding aglycones. The aqueous hydrolysed was spotted on paper chromatography plates together with authentic sugars (PC, ethyl acetate-formic acid-acetic acid–water, 10:1.1:1.1:2.6, v/v).

2.6. Spectral data

NMR spectra (^1H and ^{13}C) were recorded on a Mercury 300 and VARIAN 500 at 300 and 500 MHz for ^1H measurements and 75/125 MHz for ^{13}C measurements, respectively. CD_3OD, DMSO-d_6 and $CDCl_3$ were used as solvents. Experimental data were processed using MestRe-C software. Mass spectra were recorded on a MAT 8200 instrument with electron energy 70 eV. 3-Nitrobenzyl alcohol was used as a matrix in fast atom bombardment mass spectrometry (FAB-MS), while electrospray ionisation mass spectra of the light petroleum and chloroform fractions were recorded on a VG QUATTRO II mass spectrometer. Electrospray ionisation mass spectrometry (ESI-MS) conditions were as following: acquisition mode, ESI positive; mass scan range, 300–500 *m/z*; 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 temp. 82 °C; pressures for analyser vacuum and gas cell are 1.8e-5 and 1.5e-4 mbar, respectively. Drying and nebulising gas was nitrogen. 10 mg of light petroleum and chloroform fractions were separately dissolved in MeOH (1 mL) and 100 μL from these solutions were taken and diluted with 500 μL formic acid 2% (v/v) in acetonitrile–water (1:1, v/v). 100 μL from each diluted solution was qualitatively analysed by direct introduction into ESI positive ion mode.

2.7. LC–ESI/MS

2.7.1. Flavonoids

The chromatographic separation of the ethyl acetate fraction was carried out by HPLC using a reversed phase C-18 (RP C-18) LiChro CART (Merck-Darmstadt) column [250 × 4 mm, 5 < mu > m)]. 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: 0–60 min, gradient from 0–25% B; 60–62.5 min, gradient from 25–50% B; 62.5–70 min, isocratic at 50% B; 70–77 min, gradient from 50–100% B; and 77–87 min, isocratic at 100% B. The column was equilibrated for 10 min prior to each analysis. Flow rate 1 mL/min. MS analysis was performed under the following conditions: acquisition mode, ESI negative; nebuliser gas, N_2, 0.25 l/min; capillary, 3.00 kV, HV lens –0.50 kV; cone, –35 V; source temp., 120 °C; RF lens, –0.2 V; skimmer, –1.5 V; mass scan range, 200–800 *m/z*; interscan time 0.1 s. MS data were acquired and processed using MassLynx $V_{4.0}$ software. Stock solution of flavonoid glycoside components of the ethyl acetate fraction (10 mg residue dissolved in 1 mL DMSO). 100 μL from the stock solution was diluted with 500 μL formic acid 2% (v/v) in acetonitrile–water (1:1, v/v) and 100 μL from the diluted solution was qualitatively analysed by direct introduction to liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI/MS) in negative ion mode. Available authentic reference compounds such as hesperidin, neohesperidin, naringin, and rutin were dissolved in DMSO and the stock solutions (10 mg/mL) were stored at 4 °C and protected from daylight. Prior to injection in HPLC, stock solutions were diluted with acetonitrile–water mixture (1:1, v/v).

2.7.2. Limonoids

The chromatographic separation was carried out by HPLC using RP C-18 LiChro CART (Merck-Darmstadt) column [250 × 4 mm (5 < mu > m)]. The column was eluted at a flow rate of 1 mL/min. 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% to 70% B; 110–115 min, gradient from 70% to 100% B; 115–140 min, isocratic at 100% B. MS analysis was performed using ESI (positive ion mode) under the same conditions as mentioned in LC–ESI/MS determination of flavonoid glycosides.

2.8. Antioxidant activity

Stock solutions of the crude methanol extract and fractions (light petroleum, chloroform and ethyl acetate) were prepared by dissolving 10 mg in 1 mL methanol. Serial dilution was done from each stock solutions and 500 μL from each dilution was tested individually by adding to 500 μL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH). Standard solutions of 500 μL were prepared from stock solutions of 7 mg/mL of the pure reference compounds: hesperidin, neohesperidin, naringin, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 5-demethylnobelitin, nobelitin, and 6-demethoxynobelitin. The mixtures were vigorously shaken and allowed to stand in the dark for 30 min at room temperature. UV/VIS absorbance was measured (LKB BIOCHROM ULTROSPEC PLUS 4054) at 517 nm against blanks lacking DPPH (negative controls). The antioxidant activity, expressed as IC_{50} (μg/mL), was compared with standard antioxidants such as ascorbic acid (Ricci et al., 2005).

2.9. Lipoxygenase inhibition

Inhibition of soybean 5-lipoxygenase (5-LOX) by total methanol extract and different fractions such as light petroleum, chloroform and ethyl acetate (25 mg/mL absolute ethanol, stock solutions) was determined spectrophotometrically under the following conditions: to 970 μL of phosphate buffer (pH 9.0), were added 10 μL of 5-LOX (1 mg/mL) and 20 μL from each of the nine different dilutions of the tested samples (16–250 μg/mL from stock solutions), which was subsequently incubated at room temperature for 10 min. The enzymatic reaction was started by adding 25 μL of 62.5 mM sodium linoleate and monitored spectrophotometrically at 234 nm every 10 s for 3 min. The initial reaction rates were determined from the slope of the straight-line portion of the curve and inhibition of the enzymatic activity was calculated from triplicate experiments. Nordihydroguaiaretic acid (NDGA) was used as a positive reference compound (IC_{50} 0.2 ± 0.1 μg/mL) (Ashour et al., 2009).

2.10. Cytotoxicity

2.10.1. Cell culture

Caco-2, COS7 and HeLa cells were obtained from Prof. Dr. G. Fricker, IPMB, Heidelberg and maintained in DMEM (glutamate I) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin in addition to 1 mM sodium pyruvate, and 10 mM non-essential amino acids in case of Caco-2 cell lines. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 at 37 °C.

2.10.2. MTT assay

Sensitivity of the cells to drugs was determined in triplicate using the MTT cell viability assay (Marks, Belov, Davey, Davey, & Kidman, 1992). Exponentially growing Caco-2 (2×10^4 cells/well), COS7 and HeLa cell lines were seeded in a 96-well plate (Greiner Labortechnik®) after trypsinisation of a subconfluent culture flask. After incubation for 48 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 PBS. During incubation for further 4 h, MTT is reduced by mitochondrial dehydrogenases of viable cells to a purple

formazan product. The medium was discarded and formazan crystal dissolved in 100 μL DMSO. The plates were shaken for 15 min at room temperature, and the spectrophotometric absorbance was monitored at 570 nm using Tecan® ELISA plate reader.

2.11. Statistical analysis

All experiments were repeated at least three times. Results are reported as means ± SE. The IC_{50} 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).

3. Results and discussion

The alcoholic extract of *C. jambhiri* was fractionated using different solvents (e.g. light petroleum, chloroform, and ethyl acetate) and each fraction was chromatographed on silica gel column. Column fractions were examined by TLC and fractions having the same Rf were pooled and purified either by crystallisation or by preparative TLC. Fractionation of the extracts yielded well-known substances, most of which, however, had not yet been described as constituents of *C. jambhiri*. The chemical structures of the isolated compounds were identified as follow by mass spectrometry (MS) as well as nuclear magnetic resonance (1D- and 2D-NMR) including attached proton test (APT), H-correlation spectroscopy (H-COSY), heteronuclear multiple quantum coherence (HSQC), heteronuclear multiple bond coherence (HMBC), and nuclear overhauser effect spectroscopy (NOESY) (Fig. 1).

3.1. Compound 1

White amorphous powder; EI/MS (rel. int.): *m/z* 414 [M1, β-sitosterol]$^+$ (100), 412 [M2, stigmasterol]$^+$ (22), 396 [M1-H_2O]$^+$ (26), 381 (13), 255 (17), 213 (12). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 3.5 (1H, tt, *J* = 4.5, 11.1 Hz, H-3), 5.4 (1H, br. s, H-6), 0.7 (3H, s, H-18), 1.0 (3H, s, H-19), 0.9 (3H, d, *J* = 6.6 Hz, H-21), 5.2 (1H, dd, *J* = 8.6, 15.2 Hz, H-22), 5.0 (1H, dd, *J* = 8.7, 15.2 Hz, H-23), 0.8 (3H, d, *J* = 6.8 Hz, H-26), 0.8 (3H, d, *J* = 7.4 Hz, H-27), 0.8 (3H, t, *J* = 7.6 Hz, H-29); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm): 37.2 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.2 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.1 (C-17), 11.9 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 33.9, 138.2 (C-22), 26.1, 129.3 (C-23), 45.8, 51.2 (C-24), 29.2, 31.7 (C-25), 19.0 (C-26), 19.8 (C-27), 23.1, 24.3 (C-28), 11.9, 12.2 (C-29). Compound **1** was identified as a mixture of β-sitosterol and stigmasterol from these spectral data and physical properties (Goad & Akishisa, 1997).

3.2. Compound 2

White needle-shaped crystals; HR-ESI/MS [M+1]$^+$ at *m/z* 257, EI/MS (Rel. Int.): *m/z* 256 [M]$^+$, 213 [M-43]$^+$ (20), 199 (5), 185 (12), 169 (15), 157 (11), 143 (10), 129 (34), 115 (12), 97 (24), 73 (77), 55 (72), 43 (100) and 29 (30). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.2 (OH), 2.4 (2H, m, H-2), 1.6 (2H, m, H-3), 1.3 (2H, m, H-4), 1.3 (16H, m, H-5: 12), 1.3 (2H, m, H-13), 1.3 (2H, m, H-14), 1.3 (2H, m, H-15), 0.9 (3H, m, H-16); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 179.7 (C-1, COOH), 34.2 (C-2), 32.2 (C-14), 29.3: 29.9 (C-4:13) 24.9 (C-3), 22.9 (C-15), 14.3 (C-16). Compound **2** was identified as palmitic acid from co-TLC with authentic material and spectral data (Liu et al., 2003).

3.3. Compound 3

Yellow amorphous powder; FAB-MS [M+1]$^+$ at *m/z* 373, EI/MS (rel. int.): *m/z* 372 [M]$^+$, 357 (M-15) (100), 314 (329–15) (10), 197 (8) and 83 (5). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.9 (1H, d, *J* = 8.9 Hz, H-2′), 7.0 (1H, d, *J* = 8.9 Hz, H-3′), 7.0 (1H, d, *J* = 8.9 Hz, H-5′), 7.9 (1H, d, *J* = 8.9 Hz, H-6′), 6.6 (1H, s, H-3), 4.1, 4.0, 3.9 × 2, 3.9 (5 s, OMe at C-5, -6, -7, -8, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 177.6 (s, C-4), 162.5 (s, C-2), 161.4 (s, C-4′), 151.6 (s, C-7), 148.6 (s, C-9), 147.9 (s, C-8), 144.3 (s, C-5), 138.3 (s, C-6), 127.9 (d, C-2′), 127.9 (d, C-6′), 124.0 (s, C-1′), 115.1 (s, C-10), 114.7 (d, C-3′), 114.7 (d, C-5′), 106.9 (d, C-3, 62.5, 62.2, 62.0, 61.9, 55.7 (5 q, OMe at C-5, -6, -7, -8, -4′). Compound **3** was identified as 5,6,7,8,4′-pentamethoxyflavone (tangeretin) from these spectral data and physical properties (El-Shafae, 2002; Weber et al., 2006).

3.4. Compound 4

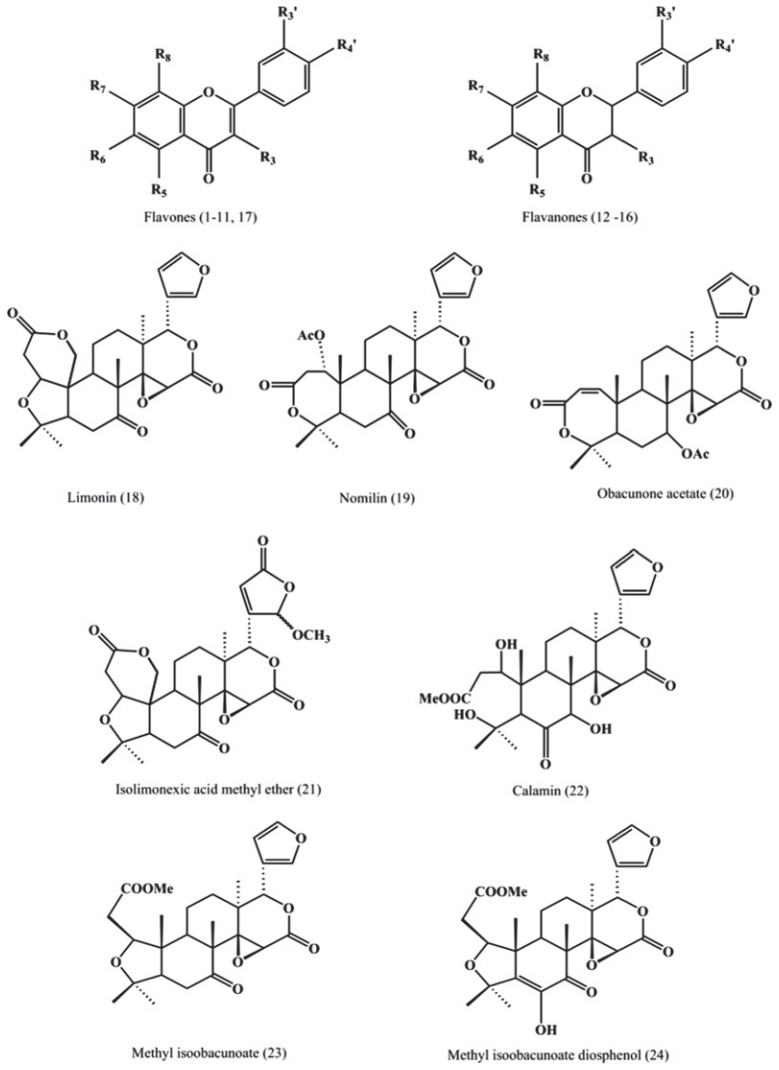
Yellowish-white needle-shaped crystals; FAB-MS [M+1]$^+$ at *m/z* 403, EI/MS (rel. int.): *m/z* 402 [M]$^+$, 387 (100), 372 (4), 357 (10), 314 (1), 197 (8) 165 (8), 162 (3), 147 (8) and 83 (0.3). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.4 (1H, d, *J* = 2.1 Hz, H-2′), 6.9 (1H, d, *J* = 8.5 Hz, H-5′) 7.6 (1H, dd, *J* = 2.1, 8.5 Hz, H-6′) 6.6 (1H, s, H-3), 4.1, 4.06, 3.9, 3.93, 3.92 × 2 (6 s, OMe at C-5, -6, -7, -8, -3′, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 177.3 (s, C-4), 160.9 (s, C-2), 151.8 (s, C-4′), 151.3 (s, C-7), 149.1 (s, C-3′), 148.3 (s, C-9), 147.6 (s, C-8), 143.9 (s, C-5), 137.9 (s, C-6), 123.9 (s, C-1′), 119.5 (d, C-6′), 114.7 (s, C-10), 111.1 (d, C-5′), 108.4 (d, C-2′), 106.8 (d, C-3), 62.2, 61.9, 61.8, 61.6, 55.9, 55.8 (6 q, OMe at C-5, -6, -7, -8, -3′, -4′). Compound **4** was identified as 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin) from these spectral data and physical properties (El-Shafae, 2002; Weber et al., 2006).

3.5. Compound 5

Dark yellow needle-shaped crystals; FAB-MS [M+1]$^+$ at *m/z* 433, EI/MS (rel. int.): *m/z* 432 [M]$^+$, 417 (100), 403 (6), 389 (4), 373 (6), 358 (5), 343 (3) 197 (10) and 165 (11). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 6.9 (1H, d, *J* = 2 Hz, H-2′), 6.8 (1H, d, *J* = 8.5 Hz, H-5′), 7.8 (1H, dd, *J* = 2, 8.5 Hz, H-6′), 4.0 × 2, 3.9 × 3, 3.8 × 2 (7 s, OMe at C-3, -5, -6, -7, -8, -3′, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 174.0 (s, C-4), 153.3 (s, C-2), 151.5 (s, C-7), 151.2 (s, C-3′), 148.9 (s, C-4′), 148.3 (s, C-5), 146.0 (s, C-9), 144.9 (s, C-6), 140.9 (s, C-3), 138.0 (s, C-8), 123.6 (s, C-1′), 122.1 (d, C-6′), 115.3 (s, C-10), 111.2 (d, C-5′), 111.1 (d, C-2′), 62.4, 62.1, 62.0, 61.8, 60.0, 56.2, 56.1 (7 q, OMe at C-3, -5, -6, -7, -8, -3′, -4′). Compound **5** was identified as 5,6,7,8,3′,4′-heptamethoxyflavone from these spectral data and physical properties (Khatoon, 1995).

3.6. Compound 6

Dark yellow needle-shaped crystals which crystallised ($CHCl_3$/MeOH); FAB-MS [M+1]$^+$ at *m/z* 419, EI/MS (rel. int.): *m/z* 418 [M]$^+$, 403 (100), 373 (6), 359 (1), 209 (5) and 165 (3). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.7 (1H, d, *J* = 2.2 Hz, H-2′), 7.0 (1H, d, *J* = 8.7 Hz, H-5′) 7.9 (1H, dd, *J* = 2.2, 8.7 Hz, H-6′), 12.4 (OH, s, H-5), 4.1 × 2, 3.9, 3.94, 3.9, 3,8 (6 s, OMe at C-3,-6,-7,-8,-3′,-4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 179.2 (s, C-4), 155.8 (s, C-2), 152.9 (s, C-7), 151.5 (s, C-3′), 149.1 (s, C-4′), 148.7 (s, C-5), 144.8 (s, C-9), 138.7 (s, C-3), 136.1 (s, C-6), 132.7 (s, C-8), 122.9 (s, C-1′), 122.3 (d, C-6′), 110.9 (d, C-5′), 110.9 (d, C-2′), 107.4 (s, C-10), 62.0, 61.7, 61.1, 60.1, 55.9 and 55.8 (6 q, OMe at C-3, -6, -7, -8, -3′, -4′). Compound **6** was identified as 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone from these spectral data and physical properties (Li, Lo, & Ho, 2006).



	Compound	R_3	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
1	5,6,7,8,4′-Pentamethoxyflavone (tangeretin)	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3
2	Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
3	3,5,6,7,8,3′,4′-Heptamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
4	5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	OCH_3	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
5	5-*O*-Demethylnobiletin	H	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
6	6-Demethoxynobiletin	H	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3
7	6-Demethoxytangeretin	H	OCH_3	H	OCH_3	OCH_3	H	OCH_3
8	5-Hydroxy-6,7,8,4′-tetramethoxyflavone	H	OH	OCH_3	OCH_3	OCH_3	H	OCH_3
9	5,4′-Dihydroxy-6,7,8,3′-tetramethoxyflavone	H	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH
10	Diosmetin 6-C-glucoside	H	OH	Glu	OH	H	OH	OH
11	Hesperidin	H	OH	H	Rut	H	OH	OCH_3
12	Neohesperidin	H	OH	H	Neo	H	OH	OCH_3
13	Narirutin	H	OH	H	Rut	H	H	OH
14	Naringin	H	OH	H	Neo	H	H	OH
15	Eriocitrin	H	OH	H	Rut	H	OH	OH
16	Neoeriocitrin	H	OH	H	Neo	H	OH	OH
17	Rutin	ORut	OH	H	OH	H	OH	OH
	Rut = rutinosides							
	Neo = neohesperidosides							

Fig. 1. Isolated and identified compounds from *C. jambhiri* peel and seeds: 1–6, 9, 11, 12, 14, 18, 19 were isolated from the peel by column chromatography; 2–9, 18 detected in ESI/MS chromatogram (positive ion mode); flavonoid glycosides 10–17 were identified by LC–ESI/MS (negative ion mode); limonoid aglycones 18–24 detected and preliminarily identified by 2/MS (positive ion mode).

3.7. Compound 7

Yellow granules; FAB-MS $[M+1]^+$ at *m/z* 389, EI/MS (rel. int.): *m/z* 388 $[M]^+$, 373 (100), 343 (5), 327 (6), 211 (14), 183 (15) and 163 (7). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.4 (1H, d, *J* = 2.2 Hz, H-2′), 6.9 (1H, d, *J* = 8.7 Hz, H-5′), 7.6 (1H, dd, *J* = 2.2, 8.7 Hz, H-6′), 6.6 (1H, s, H-3), 12.5 (OH, s, H-5), 4.1, 3.95, 3.94, 3.9, 3,8, (5 s, OMe at C-6, -7, -8, -3′, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 182.9 (s, C-4), 163.9

(s, C-2), 152.9 (s, C-7), 152.4 (s, C-4′), 149.5 (s, C-3′), 149.3 (s, C-9), 145.8 (s, C-5), 136.5 (s, C-6), 132.9 (s, C-8), 123.7 (s, C-1′), 120.1 (d, C-6′), 111.7 (d, C-5′), 108.8 (d, C-2′), 106.9 (s, C-10), 103.9 (d, C-3), 62.0, 61.7, 61.1, 56.1, 55.9 (5 q, OMe at C-6, -7, -8, -3′, -4′). Compound **7** was identified as 5-*O*-demethylnobiletin from these spectral data and physical properties (El-Shafae, 2002).

3.8. Compound 8

Yellowish-white needle crystals; FAB-MS $[M+1]^+$ at *m/z* 373, EI/MS (rel. int.): *m/z* 372.1 $[M]^+$, 357 (86), 343 (13), 328 (18), 195 (6), 167 (18), 139 (10) and 91 (3). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 7.6 (1H, dd, *J* = 2.1, 8.5 Hz, H-6′), 7.4 (1H, d, *J* = 2.1 Hz, H-2′), 6.9 (1H, d, *J* = 8.5 Hz, H-5′), 6.6 (1H, s, H-3), 6.4 (1H, s, H- 6), 3.99, 3.97, 3.95, 3.94 × 2 (5 s, OMe at C-5, -7, -8, -3′, -4′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 177.8 (s, C-4), 160.5 (s, C-2), 157.8 (s, C-5), 156.6 (s, C-7), 153.1 (s, C-9), 152.9 (s, C-3′), 150.1 (s, C-4′), 130.4 (s, C-8), 124.8 (s, C-1′), 119.5 (d, C-6′), 111.1 (d, C-5′), 109.1 (s, C-10), 108.6 (d, C-2′), 107.1 (d, C-3), 92.2 (d, C-6), 61.5, 56.5, 56.3, 56.0, 55.9 (5 q, OMe at C-5, -7, -8, -3′, -4′). Compound **8** was identified as 6-demethoxynobiletin from these spectral data and physical properties (Weber et al., 2006).

3.9. Compound 9

White needle-shaped crystals; FAB-MS $[M+1]^+$ at *m/z* 515, EI/MS (rel. int.): *m/z* 514 $[M]^+$, 333 (18), 289 (5), 255 (14), 213 (11), 201 (10), 161 (8), 135 (12), 95 (26) and 43 (53). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 5, (1H, m, H-1), 3.2 (1H, d, *J* = 15.6 Hz, H-2b), 3.1 (1H, dd *J* = 7.3 and 15.6 Hz, H-2a), 2.8 (1H, t, *J* = 15.1 Hz, H-5), 2.6 (2H, m, H-6), 2.5 (1H, dd *J* = 2.7 and 10.3 Hz, H-9), 1.6 (2H, m, H-11), 1.8 (1H, m, H- 12b), 1.1 (1H, m, H-12a), 3.8 (1H, m, H-15), 5.4 (1H, s, H-17), 6.3 (1H, s, H-21), 7.4 (1H. br. S, H-22), 7.3 (1H. br. S, H-23), 1.2 (3H, s, 18-Me), 1.3 (3H, s, 19-Me), 1.1 (3H, s, 24-Me), 1.5 (3H, s, 25a-Me), 1.5 (3H, s, 25b-Me), 2.0(3H, s, CH3-CO); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm): 206.9 (C-7), 169.2 (C-3), 169.1 (C-Ac), 166.7 (C-16), 143.2 (C-21), 140.9 (C-23), 120.1 (C-20), 109.1 (C-22), 84.3 (C-4), 77.9 (C-17), 70.7 (C-1), 65.4 (C-14), 53.4 (C-15), 52.9 (C-8), 50.9 (C-5), 44.3 (C-9), 44.2 (C-10), 38.8 (C-6), 37.5 (C-13), 35.3 (C-2), 33.4 (C-25a), 32.4 (C-25b), 32.3 (C-12), 20.8 (C-24), 20.7 (CH_3-C=O), 17.2 (C-18), 17.1 (C-19), 16.5 (C-11). Compound **9** was identified as nomilin from these spectral data and physical properties (Khalil, Maatooq, & El Sayed, 2003; Nakagawa, Duan, & Takaishi, 2001).

3.10. Compound 10

White amorphous powder; CI^+/MS $[M+1]^+$ at *m/z* 471, EI/MS (rel. int.): *m/z* 470 $[M]^+$, 329 (5), 287 (3), 241 (2), 201 (3), 187 (4), 147 (5), 136 (7), 135 (17), 108 (13) and 95 (20). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 4.1 (1H, br s, H-1), 2.2 (1H, dd, *J* = 3.2, 14.8 Hz, H-2a), 2.55 (1H, dd, *J* = 3.2, 14.8 Hz, H-2b), 2.4 (1H, dd, *J* = 2.8, 15 Hz, H-5), 2.9 (1H, dd, *J* = 2.8, 15 Hz, H-6a), 3.4 (1H, t, *J* = 15 Hz, H-6b), 2.6 (1H, dd, *J* = 2, 10 Hz, H-9), 1.8 (1H, m, H-11a), 1.5 (1H, m, H-11b), 1.4 (1H, m, H-12b), 1.8 (1H, m, H-12a), 4.1 (1H, br s, H-15), 5.4 (1H, s, H-17), 6.3 (1H, br. s, H-21), 7.4 (1H. br s, H-22), 7.2 (1H. s, H-23), 1.0 (3H, s, 18-Me), 4.8 (1H, d, *J* = 13 Hz, H-19b), 4.4 (1H, d, *J* = 13 Hz, H-19a), 1.2 (3H, s, 24-Me), 1.2 (3H, s, 25a-Me), 1.2 (3H, s, 25b-Me); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm); 208.3 (C-7), 172.7 (C-3), 168.1 (C-16), 143.2 (C-21), 141.1 (C-23), 120.4 (C-20), 109.9 (C-22), 85.3 (C-1), 85.3 (C-4), 78.6 (C-17), 69.0 (C-19), 66.1 (C-14), 53.6 (C-5), 52.8 (C-15), 50.0 (C-8), 44.8 (C-9), 43.9 (C-10), 39.2 (C-13), 37.7 (C-2), 37.7 (C-6), 32.9 (C-25a), 31.8 (C-12), 23.8 (C-25b), 20.4 (C-11), 17.6 (C-18), 17.1 (C-24). Compound **10** was identified as limonin from these spectral data and physical properties (Nakagawa et al., 2001).

3.11. Compound 11

Yellow granules; FAB-MS $[M+1]^+$ at *m/z* 375, EI/MS (rel. int.): *m/z* 374 $[M]^+$, 359 (100), 344 (9), 211 (10), 183 (9), 179 (6), 149 (5), 105 (2). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 12.5 (1H, s, OH), 7.6 (1H, dd, *J* = 2.1, 8.4 Hz, H-6′), 7.4 (1H, d, *J* = 2.1 Hz, H-2′), 7.1 (1H, d, *J* = 8.4 Hz, H-5′), 6.5 (1H, s, H-3), 4.1, 4.0, 3.96, 3.9 (4 s, OMe at C-6, -7, -8, -3′); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm); 182.9 (s, C-4), 163.9 (s, C-2), 152.9 (s, C-7), 149.5 (s, C-5), 149.4 (s, C-3′), 147.9 (s, C-4′), 145.7 (s, C-9), 136.5 (s, C-6), 132.9 (s, C-8), 123.5 (s, C-1′), 120.8 (d, C-6′), 115.1 (d, C-5′), 108.3 (d, C-2′), 106.9 (s, C-10), 103.8 (d, C-3), 62.1, 61.7, 61.1, 56.0 (4 q, OMe at C-6, -7, -8, -3′). Compound **11** was identified as 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone from these spectral data and physical properties (Li et al., 2006; Weber et al., 2006).

3.12. Compound 12

Buff granules; EI/MS (rel. int.): *m/z* 272 [M-308]+ (96), 229 (5), 191 (10), 153 (100), 147 (23), 129 (20), 107 (18) and 91 (26). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 12 (1H, s, hydrogen bonded 5-OH), 9.6 (1H, s, OH). 7.3 (2H, d, *J* = 8.4 Hz, H-2′, 6′), 6.8 (2H, d, *J* = 8.4 Hz, H-3′, 5′), 6.3 (1H, d, *J* = 2.1 Hz, H-8), 6.2 (1H, d, *J* = 2.1 Hz, H-6), 5.5 (1H, centred t, *J* = 5.4 Hz, H-2), 5.3 (1H, d, *J* = 8.2 Hz, glucosyl, H-1″), 5.2 (1H, d, *J* = 2.2 Hz, rhamnosyl, H-1‴), 3.8 (2H, m, H-6″), 3.2 (1H, m, H- 3b), 2.8 (1H, m, H- 3a), 3.2–3.7 (7H, m, rhamnoglucosyl protons) and 1.2 (3H, d, *J* = 6.6 Hz rhamnosyl CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm); 196.7 (C-4), 165.2 (C-7), 162.3 (C-5), 162.2 (C-9), 157.6 (C-4′), 128.6 (C-1′), 128.1 (C-2′), 128.1 (C-6′), 115.1 (C-3′), 115.1 (C-5′), 103.5 (C-10), 100.3 (C-1‴), 97.1 (C-1″), 96.2 (C-6), 95.0 (C-8), 78.3 (C-2), 78.2 (C-2″), 76.4 (C-3″), 76.4 (C-5″), 76.0 (C-4‴), 72.0 (C-4″), 70.2 (C-2‴), 68.9 (C-3‴), 68 (C-5‴), 60.1 (C-6″), 42.1 (C-3), 18 (C-Me). Compound **12** was identified as naringin from these spectral data and physical properties (El-Shafae, 2002; Maltese, Erkelens, Kooy, Choi, & Verpoorte, 2007).

3.13. Compound 13

White granules; FAB-MS $[M+1]^+$ at *m/z* 611, EI/MS (rel. int.): 302 $[M-308]^+$ (100), 286 (5), 271 (3), 259 (8), 179 (21), 165 (5), 137 (95), 135 (47), 129 (12) and 107 (17). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 12 (1H, s, hydrogen bonded 5-OH), 9.1 (1, H, s, OH), 6.9 (3H, m, H-2′, 5′, 6′), 6.2 (1H, d, *J* = 2 Hz, H-8), 6.1 (1H, d, *J* = 2 Hz, H-6), 5.5 (1H, dd, *J* = 7.6, 3.1 Hz, H-2), 4.9 (1H, d, *J* = 7.4 Hz, glucosyl H-1″), 4.5 (1H, d, *J* = 2.9 Hz, rhamnosyl, H-1‴), 3.8 (3H, s, OMe- 4′), 3.7 (2H, m, H-6″), 3.2–3.6 (7H, m, rhamnoglucosyl protons), 3.2 (1H, m, H- 3b) 2.9 (1H, m, H- 3a) and 1.14 (3H, m, rhamnosyl CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): d (ppm); 197.7 (C-4), 165.8 (C-7), 163.7 (C-5), 163.1 (C-9), 148.6 (C-4′), 147.1 (C-3′), 131.6 (C-1′), 118.5 (C-6′), 114.8 (C-2′), 112.7 (C-5′), 103.9 (C-10), 101.3 (C-1‴), 100.1 (C-1″), 97.0 (C-6), 96.2 (C-8), 79.1 (C-2), 76.9 (C-5″), 76.1 (C-3″), 76.1 (C-5‴), 73.6 (C-2‴), 72.7 (C-4‴), 71.4 (C-2″), 70.9 (C-3‴), 68.9 (C-4″), 66.7 (C-6″), 56.1 (C-4′-OCH_3), 42.7 (C-3), 18.5 (C-CH_3). Compound **13** was identified as hesperidin, from these spectral data and physical properties (Maltese et al., 2007) .

3.14. Compound 14

White granules; FAB-MS $[M+1]^+$ at *m/z* 611, EI/MS (rel. int.): 302 $[M-308]^+$ (28), 272 (1), 259 (3), 231 (2), 217 (2), 191 (9), 173 (11), 153 (58), 147 (21), 137 (100), 129 (18), 124 (20) and 107 (15). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 12 (1H, s, hydrogen bonded, 5-OH), 9.1 (1H, s, OH), 6.95 (1H, d, *J* = 2.1 Hz, H-2′), 6.92 (1H, dd, *J* = 2.2, 8.7 Hz, H-6′), 6.9 (1H, d, *J* = 8.7 Hz), 6.2 (1H, d,

J = 2, H-8), 6.1 (1H, d, J = 2, H-6), 5.5 (1H, dd, J = 12.6, 3.2 Hz, H-2), 5.1 (1H, d, J = 7.4 Hz, glucosyl, H-1″), 5.1 (1H, d, J = 2.8 Hz, rhamnosyl, H-1‴), 3.8 (3H, s, OMe- 4′), 3.6 (2H, m, H-6″) 3.2–3.6 (8H, m, rhamnoglucosyl protons), 3.1 (1H, m, H- 3b), 2.8 (1H, m, H-3a) and 1.2 (1H, d, J = 6 Hz rhamnosyl CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm); 197.2 (C-4), 164.8 (C-7), 162.9 (C-5), 162.7 (C-9), 147.9 (C-4′), 146.5 (C-3′), 130.8 (C-1′), 117.9 (C-6′), 115.2 (C-2′), 114.2 (C-5′), 100.4 (C-10), 101.3 (C-1‴), 97.4 (C-1″), 96.3 (C-6), 95.2 (C-8), 78.7 (C-2), 78.4 (C-5″), 77.6 (C-2‴), 76.9 (C-4‴), 76.2 (C-3″), 76.1 (C-5‴), 70.4 (C-2″), 69.6 (C-3‴), 68.3 (C-4″), 60.4 (C-6″), 55.7 (C-4′-OCH_3), 42.2 (C-3), 18.0 (C-CH_3). Compound **14** was identified as neohesperidin from these spectral data and physical properties (Maltese et al., 2007) .

A mixture of sitosterol and stigmasterol (**1**) and palmatic acid (**2**) were isolated and identified from the petroleum ether fraction of the peel.

A total of seven polymethoxyflavones were isolated and identified from the petroleum ether and chloroform fractions of the peel including: tangeretin (**3**), nobiletin (**4**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**5**), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**6**), 5-*O*-demethylnobeletin (**7**), 6-demethoxynobiletin (**8**), and 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**11**). From this class of compounds, only tangeretin had previously been reported from this plant (Chaliha et al., 1965).

Nomilin (**9**) and limonin (**10**) were isolated from the chloroform extract of the peel in a considerable yield. Limonin was the most abundant natural product. This is the first report of the isolation of limonoids from this plant, although they are common in others *Citrus* species.

Three flavanone glycosides were isolated from the ethyl acetate fraction including naringin (**12**), hesperidin (**13**) and neohesperidin (**14**). Hesperidin was the most abundant and naringin was the least abundant flavanone in the peel. Hesperidin and neohesperidin had previously been identified by HPLC in the juice of this species (Arriaga & Rumbero, 1990).

3.15. ESI/MS

Prior to the isolation of the compounds by column chromatography, MS fingerprints of the light petroleum and chloroform fractions were performed using ESI/MS. Fig. 2a reveals five major peaks in the light petroleum fraction with the following molecular masses $[M+H]^+$ at m/z 373.1, 389.1, 403.1, 419.1, and 433.3 which are attributed to the following compounds: tangeretin, 5-*O*-demethylnobiletin, nobiletin, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone and 3,5,6,7,8,3′,4′-heptamethoxyflavone, respectively. In case of the chloroform fraction, the ESI/MS spectral profile (Fig. 2b) shows many compounds with the following molecular masses $[M+H]^+$: 343.1, 359.1, 373.1, 375.1, 389.2, 403.1, 419.2, 433.3, and 471.2. These molecular ions are apparently related to the following compounds: 6-demethoxytangeretin, 5-hydroxy-6,7,8,4′-tetramethoxyflavone, 6-demethoxynobiletin, 5,4′-dihydroxy 6,7, 8,3′-tetramethoxyflavone, 5-*O*-demethylnobiletin, nobiletin, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 3,5,6,7,8, 3′,4′-heptamethoxyflavone and limonin, respectively. All of the detected compounds were confirmed later by direct analysis of the isolated compounds from the column by ESI/MS and by matching each ion peak in the total ion chromatogram with those of individual secondary metabolites.

3.16. LC–ESI/MS

Liquid chromatography (LC) combined with electrospray ionisation mass spectrometry (ESI/MS) proves to be very powerful for flavonoid characterisation (Careri, Elviri, & Mangia, 1999). Fig. 3 shows the total ion current chromatogram (TIC) obtained by LC–ESI/MS (negative ion mode) of the ethyl acetate fraction. It has been previously reported that rutinosides tend to fragment easier than the neohesperidoside, and a possible explanation is that the free hydroxyl group can be involved in breaking the β-bond between the aglycone and the sugar (Cappiello et al., 1999; Cuyckens, Ma, Pocsfalvi, & Cleays, 2000). Another trend is that rutinoside isomers elute before neohesperidoside (Cuyckens et al., 2000). Our results are in agreement with previous reports. A total of eight flavonoid glycosides, i.e. eriocitrin, neoeriocitrin, rutin, diosmetin 6-C-glucoside, narirutin, naringin, hesperidin and neohesperidin were identified. Identification of these compounds was based on comparison with authentic standards, relative retention times and literature mass data (Cappiello et al., 1999; Cuyckens et al., 2000). The MS spectra of all rutinosides (i.e. eriocitrin, rutin, narirutin and hesperidin) showed the addition of a pseudomolecular ion and a signal corresponding to the aglycone ion, while the neohesperidosides (neoeriocitrin, naringin, neohesperidin) show only a pseudomolecular ion. In the full mass scan analysis of the ethyl acetate fraction, a signal at m/z 595 was found as $[M-H]^-$. MS-MS provided a fragment ion at m/z 287 $[M-H\text{-rhamnose-glucose}]^-$ corresponding to the aglycone of eriocitrin in negative ion mode which had not previously been identified. ESI-MS ion chromatogram analysis showed a deprotonated molecule in the negative mode at m/z 579 and fragment ion by MS-MS at m/z 271. The molecular ion and aglycone correspond to narirutin which is here reported for the first time in this species. ESI/MS showed two similar deprotonated molecules at m/z 609 at different retention times. MS revealed an aglycone ion at m/z 301 and the (1‴→6″) interglycosidic linkage was detected. The one appears to be hesperidin and the other neohesperidin. Another deprotonated molecule at m/z 609 with a retention time of around 39 min is related to rutin.

LC–ESI/MS analysis of the chloroform extract of the defatted seeds resulted in the identification of seven limonoid aglycones: limonin, nomilin, calamine, obacunone acetate, methyl isoobacunoate, methyl isoobacunoate diosphenol and isolimonexic acid methyl ether. Identification of limonin and nomilin was based on the comparison of the retention times with authentic standards, chromatographic and reported MS data (Manners & Hasegawa, 1999). High resolution mass spectrometry (HRMS) data (EI mode) from limonin and nomilin have been reported (Tian & Schwartz, 2003). These data establish that all of these limonoids display a fragment at m/z 95 that is associated with the formation of a furanal radical from the limonoid furan moiety and oxygen at C-17. A further fragment ion $[M-123]^+$ arises from the loss of carbon monoxide at C-16 (28 mass units) from the $[M-95]^+$ fragment ion. The detection and identification of other limonoid aglycones in the chromatogram are based on comparison with literature data (Manners & Hasegawa, 1999).

3.17. Antioxidant activity

The DPPH assay is based on measuring the scavenging ability of antioxidants towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The free radical DPPH* is reduced to the corresponding hydrazine when it reacts with hydrogen donors. The DPPH assay is considered a valid and easy assay to evaluate the antioxidant activity of natural products. The IC_{50} values of the pure isolated compounds are given in Table 1. Some of the tested compound (Naringin, 7 mg/mL) was not completely soluble in MeOH and therefore a binary solvent of MeOH and DMSO (1:1, v/v) (MD) was used instead. In order to compare the activities of MeOH-soluble and MeOH-insoluble compounds, a relation coefficient of rutin (1.3) was calculated by determining the IC_{50} values of rutin in MeOH (2.8) and in MD (2.2). Table 1 lists data displaying the ability of the chloroform fraction to scavenge DPPH*, with a stronger scavenging effect than the other fractions. Of the tested

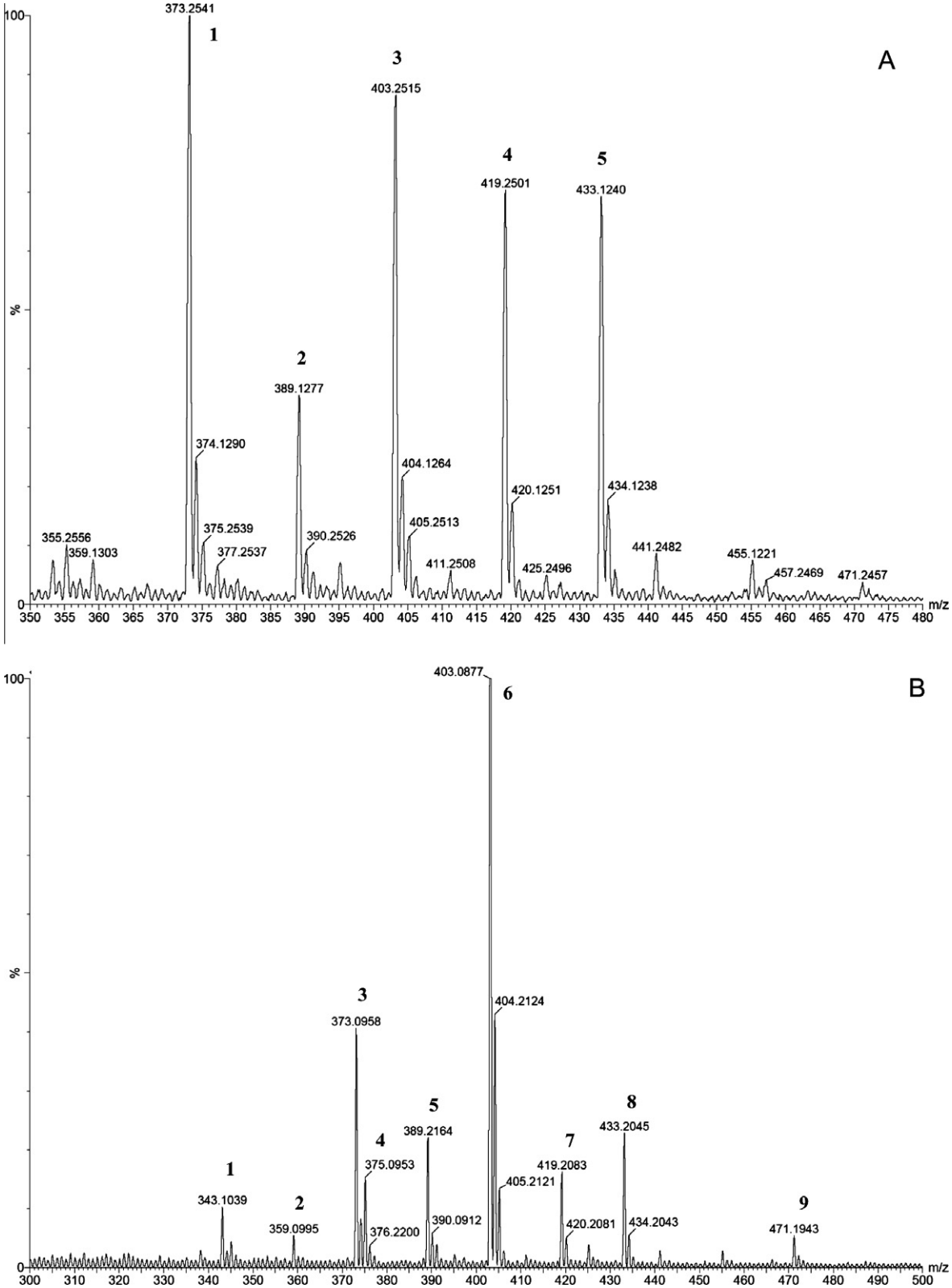


Fig. 2. ESI/MS profiles from *C. jambhiri* (A) petroleum ether fraction: tangeretin (**1**), 5-*O*-demethylnobiletin (**2**), nobiletin (**3**), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**4**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**5**); and (B) chloroform fraction: 6-demethoxytangeretin* (**1**), 5-hydroxy-6,7,8,4′-tetramethoxyflavone* (**2**), 6-demethoxynobiletin (**3**), 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**4**), 5-*O*-demethylnobiletin (**5**), nobiletin (**6**), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**7**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**8**), and limonin (**9**); *tentatively identified by ESI/MS.

compounds, rutin and quercetin exerted the strongest activity and these compounds were used as positive controls. Neohesperidin is more active than hesperidin, while naringin has only minor activity. Total polymethoxyflavones from the petroleum ether fraction (5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 5-*O*-demethylnobelitin, nobelitin and 6-demethoxynobelitin) together exerted

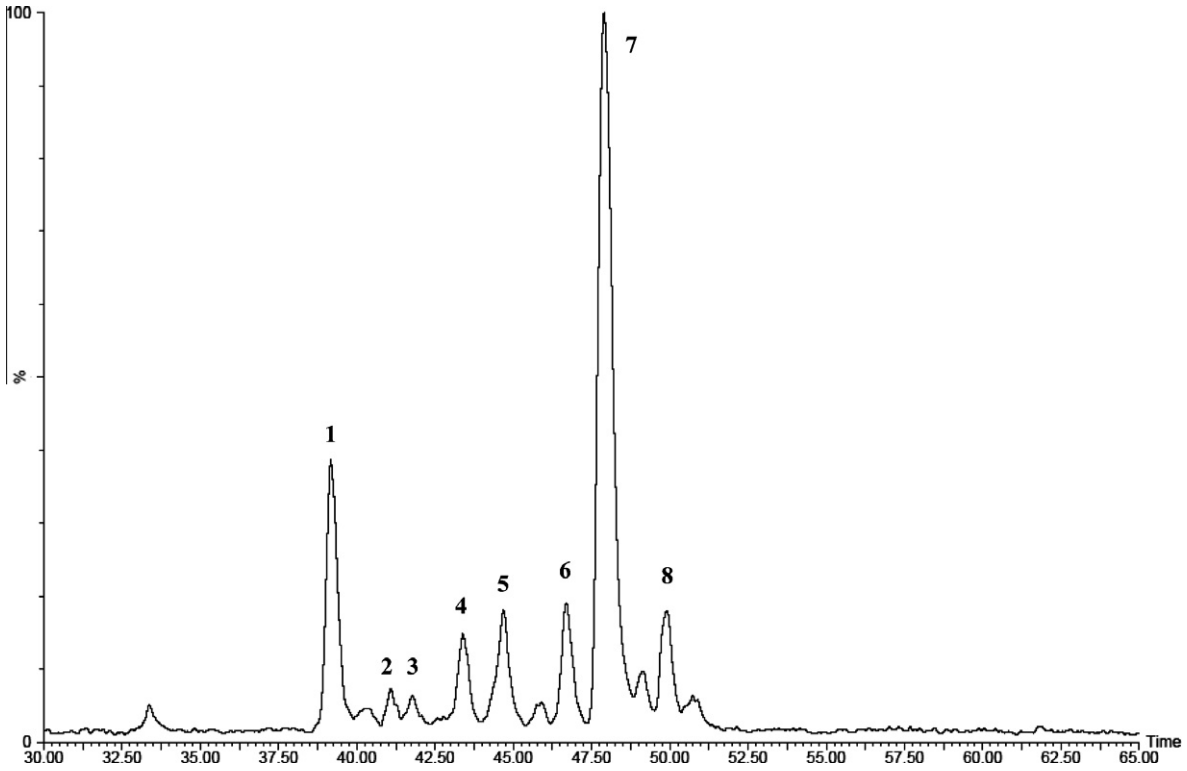


Fig. 3. LC–ESI/MS (negative ion mode) of the ethyl acetate fraction from *C. jambhiri*. Eriocitrin (**1**), neoeriocitrin (**2**), rutin (**3**), diosmetin 6-C-glucoside (**4**), narirutin (**5**), naringin (**6**), hesperidin (**7**), and neohesperidin (**8**).

Table 1
DPPH* scavenging activity of fractions and isolated compounds from *C. jambhiri*.

Sample	IC_{50} (μg/mL)
Fractions	
Total extract	223.5 ± 0.5
Petroleum ether fraction	166.6 ± 1.1
Chloroform fraction	119.4 ± 0.8
Ethyl acetate fraction	171.3 ± 1.3
Isolated compounds	
Hesperidin	361.5 ± 0.4
Neohesperidin	178.9 ± 0.4
5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	735.6 ± 0.4
5-Demethylnobelitin	752.3 ± 0.2
Nobiletin	3557.1 ± 0.5
6-Demethoxynobelitin	1284.2 ± 0.5
Positive control	
Ascorbic acid	16.3 ± 0.2
Rutin	2.2 ± 0.3
Quercetin	3.7 ± 0.3

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

a lower IC_{50} than the individual compounds, thus pointing to a synergistic effect (Table 1).

3.18. Lipoxygenase inhibition

The inhibition of 5-lipoxygenase (5-LOX) is currently a subject of intense research targeted towards the discovery of novel anti-allergic and anti-inflammatory agents. The prime objective of this study was therefore to evaluate the ability of total extracts and different fractions to inhibit 5-LOX *in vitro*. Previous studies have shown that nordihydroguaiaretic acid (NDGA) could be used as a reference compound for studies of 5-LOX inhibition, due to its widely reported strong inhibitory activity on this enzyme (Abad, Bermejo, & Villar, 1995). Table 2 summarises the results obtained from total extracts and different fractions and IC_{50} values for 5-LOX: the activity of the studied species of 5-LOX inhibitors decreased from petroleum ether, followed by chloroform, ethyl acetate and total extract of the peel as shown.

3.19. Cytotoxicity

Table 3 summarises data on the IC_{50} of the tested samples against Caco-2, HeLa and COS7 cell lines. All tested samples show high IC_{50} values in the chemotherapy resistant Caco-2 cell line in comparison to the sensitive HeLa and COS7, which have a low endogenous ABCG2 and P-gp expression. The Caco-2 cell line highly expresses multidrug resistance ABC-transporter genes (El-Readi, Hamdan, Farrag, El-Shazly, & Wink, 2010). In addition, the polymethoxyflavones are most cytotoxic against COS7, while 5-*O*-demethylnobiletin shows a stronger cytotoxic effect in COS7 and Caco-2 cell lines than nobiletin. This may be due to the free phenolic hydroxyl group in 5-*O*-demethylnobiletin which forms ionic bonds with charged amino acid residues. These ionic bonds are stable and reduce the structural flexibility of proteins. Polyphenols interfere in an unselective manner with proteins in that the phenolic hydroxyl groups can partly dissociate under physiological conditions resulting in phenolates. These form stable ionic bonds with the positively charged side chains of basic amino acids. All polyphenols can also form hydrogen bonds with electronegative atoms of proteins. One single non-covalent bond is relatively weak, but if several bonds are formed, a change in protein conformation is likely to occur and can lead to changes in protein activity (Wink, 2008). In case of the HeLa cell line, nobiletin shows a stronger cytotoxic effect than 5-*O*-demethylnobiletin. As hesperidin and neohesperidin are isomers, it is not surprising that the IC_{50} values are very similar in case of COS7, while in case of the HeLa cells hesperidin is more cytotoxic than neohesperidin and the reverse being

Table 2
Inhibition of 5-lipoxygenase by fractions of *C. jambhiri* (means ± SD) in %.

Conc. (μg/mL)	Total extract	Petroleum ether fraction	$CHCl_3$ fraction	Ethyl acetate fraction
16	4.3 ± 0.1	20.4 ± 0.5	9.3 ± 0.5	4.0 ± 0.8
23	4.9 ± 0.1	44.9 ± 0.4	14.1 ± 0.5	4.9 ± 1.3
31	7.2 ± 0.1	63.1 ± 1.8	29.0 ± 0.8	11.4 ± 1.3
46	8.5 ± 0.3	81.0 ± 0.8	43.2 ± 1.5	16.9 ± 1.2
63	14.4 ± 0.4	92.7 ± 1.6	70.7 ± 1.3	30.6 ± 1.3
94	19.2 ± 0.2	99.6 ± 0.5	92.8 ± 1.6	54.3 ± 2.6
125	60.7 ± 1.9	99.6 ± 0.5	99.6 ± 0.5	79.3 ± 2.9
188	97.2 ± 0.5	99.4 ± 0.4	99.6 ± 0.5	97.1 ± 0.8
250	99.5 ± 0.6	99.7 ± 0.5	99.6 ± 0.6	99.5 ± 1.5
IC_{50}	124.7 ± 2.4	29.0 ± 1.0	48.7 ± 1.8	93.7 ± 2.7

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

Table 3
Cytotoxicity of isolated compounds from *C. jambhiri* against Caco-2, HeLa and COS7 cell lines; data represent IC_{50} values (μM) mean ± SD.

Compound	Caco-2	HeLa	COS7
Nobiletin	172.3 ± 19.2	22.9 ± 4.8	4.9 ± 0.2
5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	137.4 ± 44.7	48.2 ± 28.6	4.2 ± 0.4
5-*O*-Demethylnobiletin	44.9 ± 6.1	101.1 ± 16.8	3.8 ± 0.4
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
Acetoside	294.2 ± 20.1	78.5 ± 3.6	144.5 ± 15.5

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

true in the case of Caco-2 cell lines. Both of them contain more than 6 phenolic hydroxyl groups which form ionic bonds with charged amino acid residues of proteins as discussed above. The sterol mixtures have higher activity in COS7 followed by HeLa cells and Caco-2 cell lines. The cytotoxic activity could be due to the sterol mixtures being more lipophilic and interacting with the lipophilic side chains of phospholipids or cholesterol, in turn affecting membrane fluidity and disrupting proper membrane function by changing the three-dimensional conformation of membrane proteins (Wink, 2008).

Acknowledgements

We gratefully acknowledge Theodor C. H. Cole for consultations regarding the manuscript and for English proofreading. Heiko Rudy and Tobias Timmermann of IPMB (Dept. of Bioorganic Chemistry), University of Heidelberg, are thanked for providing MS and NMR measurements. Astrid Backhous for technical assistance. D., Hamdan and M. Z., El-Readi are grateful for financial support by the Egyptian Ministry of Higher Education and Scientific Research.

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