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Flavonoid investigation, LC–ESI-MS profile and cytotoxic activity of *Raphanus raphanistrum* L. (*Brassicaceae*)

Lamyaa F. Ibrahim[1], Ahmed Elkhateeb[1*], Mona M. Marzouk[1], Sameh R. Hussein[1], El-Sayed S. Abdel-Hameed[2,3] and Mona E. S. Kassem[1]

[1]*Department of Phytochemistry and Plant Systematics, National Research Centre, 33 El Bohouth St., Dokki, Giza, Egypt*
[2]*Laboratory of Medicinal Chemistry, Theodor Bilharz Institute, Giza, Egypt*
[3]*Laboratory of Natural Products Analysis, Faculty of Science, Taif University, Saudi Arabia*

ABSTRACT

*Raphanus raphanistrum L. (Family: Brassicaceae) is an important crop used worldwide for its medicinal and therapeutic properties as well as its high nutritional value. The present work aims to investigate the flavonoid constituents and the cytotoxicity for R. raphanistrum extract. Ten flavonoid compounds were isolated by chromatographic techniques They were identified as kaempferol (**1**), kaempferol 3-O-α-arabinopyranoside (**2**), kaempferol 3-O-α- rhamnopyranoside (**3**), kaempferol 3-O-β-glucopyranoside-7-O-α-rhamnopyranoside (**4**), kaempferol 3-O-α-rhamnopyranoside-7-O-β-glucopyranoside (**5**), kaempferol 3,7-di-O-α-rhamnopyranoside (**6**), kaempferol 3-O-(2˝–β-glucopyranosyl)-α-rhamnopyranoside-7-O-α-rhamnopyranoside (**7**), quercetin 3-O-α-rhamnopyranoside-7-O-α-arabinopyranoside (**8**), quercetin 3-O-β-glucopyranoside-7-O-α-rhamnopyranoside (**9**), quercetin 3-O-α-rhamnopyranoside-7-O-β-glucopyranoside (**10**). Except for compounds **4** and **5**, all flavonoids were firstly isolated from the plant. The LC-ESI-MS technique was also performed and revealed the tentative identification of kaempferol and quercetin nuclei with high grade of glycosylation for the first time. Furthermore, moderate cytotoxic activity, for R. raphanistrum extract, against HEPG2 and MCF7 at 100 mg/mL were assisted with cell viability 62.4% and 57.9%, respectively.*

Keywords: *Raphanus raphanistrum*, Brassicaceae, Flavonoids, LC–ESI-MS, Cytotoxic activity.

INTRODUCTION

Raphanus L. is a genus of the Brassicaceae family, comprising four species mainly distributed in Europe, Mediterranean region to Central Asia [1]. In Egypt, it is represented by a monotypic wild species; *Raphanus raphanistrum* L., commonly named as “radish” [2]. Ancient Egyptians and Romans used the root crop as valuable food [3].

The seeds of most *Raphanus* species contain high percentages of essential oil, calcium, iron, magnesium, ascorbic acid, folic acid, vitamin B6, riboflavin and low percentages of saturated fats and cholesterol [4].

The leaves are good source of protein, amino acids and enzymes. *R. raphanistrum* is used worldwide for its culinary and medicinal properties especially as it has cardiovascular inhibitory effects mediated through activation of muscarinic receptors, so it is used in hypertension [5]. In addition, it has a potent hepatoprotective effect [6], good hyperglycemic efficiency and anti-inflammatory activities as well as immunomodulatory activity both *in vitro* and *in vivo* of the extract [7].

R. raphanistrum is an important source of antimicrobial substances against *Sytaphylococcus aureus, Bacillus subtilis* and *Escherichia coli* [8]. Also, the seeds are reported to have a broad spectrum antibiotic activity especially against *Mycobacterium tuberculosis* [9]. Additionally, the whole plant showed substantial antifungal activity against several fungal species with high degree of specificity to filamentous fungi [9]. Chemopreventive efficacy of *R. raphanistrum* extract is reported to induce apoptosis and inhibit cell proliferation in human cancer cells.

Chemical constituents profile showed the identification of alkaloid and nitrogenous compounds [11-12-13]. Hydroxycoumarins aesculetin and scopoletin were also identified [14]. From the steam volatile constituents of fresh radish, 1- methylthio-3-pentanone, pentyl hexyl, dimethyl disulfide, methyl methanethiolsulfinate and 4-methyl pentyl isothiocyanate were isolated as major components [15]. Radish contains organic acids (oxalic, malic, malonic, and erythorbic acids) [16] and phenolic acids (gentisic, salicylic, vanillic, hydroxycinnamic, *p*-hydroxybenoic, *p*-coumaric, caffei and ferulic acids) [14]. The 7-*O*-rhamnoside of kaempferol, quercetin and isorhamnetin, the 3-*O*-rhamnoside-7-*O*-glucoside of kaempferol and quercetin, as well as kaempferol-3-*O*-glucoside 7-*O*-rhamnoside and quercetin-7-O-arabinoside-3-*O*-glucoside were isolated from *R. raphanistrum* [17]. It also has a high content of aglycones; apigenin, luteolin, myricetin, kaempferol and quercetin [18].

Liquid chromatography (LC) hyphenated with different types of detectors are methods of choice for analysis of polar, thermo labile compounds with high molecular masses. The present study demonstrates the applicability of LC; that separates the sample components; combined with mass spectrometric detectors for definite quantification and identification of flavonoid conjugates assigned for *R. raphanistrum*.

EXPERIMENTAL SECTION

General

NMR experiments were recorded on a Jeol EX-500 spectroscopy: 500 MHz (^{1}H NMR), 125 MHz (^{13}C NMR). UV spectrophotometer (Shimadzu UV-240), EIMS: Finnigan-Mat SSQ 7000 spectrometer. Column chromatography (CC) Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, Seelze Hanver, Germany) and Sephadex LH-20 (Pharmazia) using MeOH/H_2O as eluent. Paper chromatography (PC) (descending) Whatman No. 1 and 3 MM papers, using solvent systems 1) H_2O, 2) 15% HOAc (H_2O–HOAc 85:15), 3) BAW (*n*-BuOH–HOAc–H_2O 4:1:5, upper layer), 4) (C_6H_6-*n*-BuOH-H_2O-pyridine 1:5:3:3, upper layer). Solvents 3 and 4 were used for sugar analysis. Complete acid hydrolysis (2 N HCl, 2 h, 100°C) was carried out and followed by paper co-chromatography with authentic samples to identify the aglycones and sugar moieties [19, 20]. Authentic samples were obtained from our research team (Department of phytochemistry and plant systematics, NRC).

*Kaempferol (**1**) [21]*

Yellow amorphous powder, R_f: 0.74 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266,292sh, 319sh, 366; (+NaOMe), 276, 320sh, 411; (+$AlCl_3$) 269, 305, 350, 423; (+$AlCl_3$/HCl) 267, 305, 350, 424; (+NaOAc) 274, 306, 378; (+NaOAc/H_3BO_3) 265, 294, 319, 369.^{1}H NMR (500 MHz, DMSO-*d6*, δ, ppm, *J*/Hz): 8.03 (2H, d, *J*= 8.5, H-2′,6′); 6.93 (2H,d, *J*= 8.5, H-3′,5′); 6.41 (1H, d, *J*= 2.0, H-8); 6.17(1H,d, *J*= 2.0, H-6). EI-MS: *m/z* 286

*Kaempferol 3-O-α-arabinopyranoside (**2**) [22]*

Yellow amorphous powders, R_f: 0.66 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266, 346; (+NaOMe) 274, 327sh, 401; (+$AlCl_3$) 274, 304, 349, 396; (+$AlCl_3$/HCl) 274, 345, 394; (+NaOAc) 274, 305, 393; (+NaOAc/H_3BO_3) 267, 352. ^{1}H NMR (500 MHz, DMSO-d6, δ, ppm, *J*/Hz): 8.02 (2H,d, *J*= 8.5, H-2′,6′); 6.85 (2H,d, *J*= 8.5, H-3′,5′); 6.37 (1H,d, *J*= 2.0, H-8); 6.19(1H,d, *J*= 2.0, H-6); 5.38 (1H,d, *J*=6.9, H-1″); 3-4 (4H, m, overlapped with -OH signals, H-2″-H-5″). ESI-MS: *m/z* 417 [M-H]$^-$.

*Kaempferol 3-O-α-rhamnopyranoside (**3**) [19, 23]*

Yellow amorphous powder, R_f: 0.69 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266, 348; (+NaOMe) 274, 327sh, 401; (+$AlCl_3$) 274, 304, 349, 398; (+$AlCl_3$/HCl) 274, 345, 394; (+NaOAc) 274, 305, 395; (+NaOAc/H_3BO_3) 267, 352. ^{1}H NMR (500 MHz, DMSO-*d6*, δ, ppm, *J*/Hz): 8.03 (2H, d, *J*= 8.5, H-2′,6′); 6.93 (2H,d, *J*= 8.5, H-3′,5′); 6.41 (1H, d, *J*= 2.0, H-8); 6.17 (1H,d, *J* = 2.0, H-6); 5.3 (1H, d, *J*= 2, H-1‴); 3-4 (m, sugar protons overlapped with -OH proton signals); 0.84 (3H, d, *J* = 6.0, CH_3-rhamnose at position 3). ESI-MS: *m/z* 431 [M-H]$^-$.

*Kaempferol 3-O-β-glucopyranoside-7-O-α-rhamnopyranoside (**4**) [24]*

Yellow amorphous power, R_f: 0.48 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266, 350; (+NaOMe) 258, 266, 401; (+$AlCl_3$) 274, 300, 350, 398; (+$AlCl_3$/HC1) 274, 299, 349, 399; (+NaOAc) 265, 358, 399; (+NaOAc/H_3BO_3) 257sh, 265, 352. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 8.04 (2H, d, *J*= 8.5, H-2′,6′); 6.87 (2H, d, *J*= 8.5, H-3′,5′); 6.8 (1H, d, *J*=1.8, H-8); 6.41 (1H, d, *J*= 1.8, H-6); 5.52 (1H, d, *J*=2.0, H-1‴); 5.46 (1H, d, *J*= 7.5, H-1″); 3-4 (m, sugar protons overlapped with -OH proton signals); 1.1 (3H, d, *J* = 6.0, H-6‴). ^{13}C NMR (125 MHz, DMSO-d_6,

δ, ppm): 178.2 (C-4), 162.1 (C-7); 161.4 (C-5); 160.7 (C-4′); 157.3 (C-2), 156.5 (C-9), 135.9 (C-3), 131.5 (C-2′, C-6′), 121.1 (C-1′), 115.7 (C-3′, C-5′), 106.2 (C-10), 101.2 (C-1″),100 (C-6), 98.9 (C-1‴), 94.9 (C-8), 78.1 (C-3″), 76.9 (C-5″), 74.7 (C-2″), 72.5 (C-4″), 70.7 (C-2‴), 70.6 (C-3‴), 70.4 (C-5‴), 70.1 (C-4‴), 61.2 (C-6″), 18.4 (C-6‴). ESI-MS: *m/z* 593 $[M-H]^-$.

*Kaempferol 3-O-α-rhamnopyranoside-7-O-β-glucopyranoside (**5**) [24]*
Yellow amorphous power, R_f 0.47 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266, 349; (+NaOMe) 258, 266, 401; (+$AlCl_3$) 274, 300, 350, 399; (+$AlCl_3$/HC1) 274, 299, 349, 399; (+NaOAc) 265, 358, 399; (+NaOAc/H_3BO_3) 265, 350. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 7.9 (2H, d, *J*= 8.5, H-2′,6′); 6.9 (2H, d, *J*= 8.5, H-3′,5′); 6.78 (1H, d, *J*=1.8, H-8); 6.45 (1H, d, *J*= 1.8, H-6); 5.33 (1H, d, *J* =2, H-1″); 5.02 (1H, d, *J*=7.5 , H-1‴); 3-4 (m, sugar protons overlapped with -OH proton signals); 0.88 (3H, d, *J*= 6.0, H-6″). ESI-MS: *m/z* 593 $[M-H]^-$.

*Kaempferol 3,7-di-O-α-rhamnopyranoside (**6**) [21]*
Whitish yellow crystals, R_f 0.52 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 266, 345; (+NaOMe) 266, 387; (+$AlCl_3$) 274, 300, 345, 399; (+$AlCl_3$/HC1) 274, 299, 341, 399; (+NaOAc) 265, 354, 389; (+NaOAc/H_3BO_3) 266, 314, 243. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 7.76 (2H, d, *J*= 9.0, H-2′,6′); 6.87 (2H, d, *J*= 9.0, H-3′,5′); 6.66 (1H, d, *J*=1.8, H-8); 6.35 (1H, d, *J*= 1.8, H-6); 5.5 (1H, d, *J*=2, H-1‴); 5.3 (1H, d, *J*= 2, H-1″); 3-4 (m, sugar protons overlapped with -OH proton signals); 1.1 (3H, d, *J*= 6.0, H-6″; for rhamnose at position 7); 0.8 (3H, *J*= 6.0, H-1‴; for rhamnose at position 3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 178 (C-4), 162.3 (C-7); 161.5 (C-5); 160.7 (C-4′); 158.3 (C-2), 156.6 (C-9), 135 (C-3), 131.2 (C-2′, C-6′), 121 (C-1′), 116 (C-3′, C-5′), 106.3 (C-10), 102.4 (C-1″),100 (C-6), 99.03 (C-1‴), 95 (C-8), 72.2 (C-4″), 71.7 (C-5″), 71.2 (C-4‴), 70.9 (C-2″), 70.8 (C-2‴), 70.6 (C-3″), 70.6 (C-3‴), 70.3 (C-5‴), 18.00 (C-6″), 18.5 (C-6‴). ESI-MS: *m/z* 577 $[M-H]^-$.

*Kaempferol 3-O-(2″– β-glucopyranosyl)- α- rhamnopyranoside-7-O-α-rhamnopyranoside (**7**) [21]*
Pale yellow crystals, R_f 0.37 (BAW). UV spectral data, λ_{max}(nm): (MeOH): 266, 342; (+NaOMe) 266, 390; (+$AlCl_3$) 274, 300, 345, 393; (+$AlCl_3$/HCl) 275, 301, 344, 394; (+NaOAc) 264, 382; (NaOAc/H_3BO_3) 265, 343. ^{1}H NMR (500 MHz, DMSO-*d6*, δ, ppm, *J/Hz*): 7.78 (2H, d, *J*= 8.5, H-2′,6′); 6.85 (2H, d, *J*= 8.5, H-3′,5′); 6.64 (1H, d, *J*= 2.0, H-8); 6.35 (1H, d, *J*= 2.0, H-6); 5.48 (1H, d, *J*= 2.0, H-1‴); 5.33 (1H, d, *J*= 2.0, H-1″); 4.16 (1H, d, *J*=7.00, H-1⁗); 3.4 (m, sugar protons overlapped with -OH proton signals); 1.12 (3H, d, *J*= 6, H-6‴; rhamnose at position 7); 0.87 (3H, d, *J*= 6, H-6″; rhamnose at position 3). ^{13}C NMR (125 MHz, DMSO-*d6*, δ, ppm): 177.4 (C-4), 162.6 (C-7), 162.2 (C-5), 161.3 (C-4′), 156.6 (C-9), 157.1 (C-2), 134.8 (C-3), 131.2 (C-2′, C-6′), 121 (C-1′), 116.3 (C-3′, C-5′), 105.9 (C-1‴), 106.75 (C-10), 101.3 (C-1″), 100.1 (C-6), 99.9 (C-1‴), 95.1 (C-8), 81.4 (C-2″), 79 (C-3⁗), 76.7(C-5‴), 76.2 (C-2⁗), 74.1 (C-4″), 72.3 (C-4⁗), 71.1 (C-3″), 70.9 (C-5″), 70.7 (C-4‴), 70.5 (C-3‴), 70.3 (C-2‴), 69.9 (C-5‴), 61.9 (C-6⁗), 18.4 (C-6″), 18.1 (C-6‴). ESI-MS: *m/z* 739 $[M-H]^-$.

*Quercetin 3-O-α -rhamnopyranoside-7-O-α-arabinopyranoside (**8**) [19, 23]*
Yellow amorphous power, R_f 0.47 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 258, 295sh, 356 (+NaOMe) 266, 406; (+$AlCl_3$) 272, 300sh, 340sh, 426; (+$AlCl_3$/HC1) 270, 299sh, 358, 401; (+NaOAc) 259, 396sh, 367, 416; (+NaOAc/H_3BO_3) 261, 295sh, 377. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 7.59 (2H, m, H-2′,6′); 6.9 (1H, d, *J*= 8.5, H-5′); 6.8 (1H, d, *J*=2.0, H-8); 6.48 (1H, d, *J*= 2.0, H-6); 5.4 (1H, d, *J*=2.0, H-1″); 5.02 (1H, d, *J*= 7.0, H-1‴); 3-4 (m, sugar protons overlapped with -OH proton signals); 0.82 (3H, d, *J*= 5.8, H-6″). ESI-MS: *m/z* 579 $[M-H]^-$.

*Quercetin 3-O-β-glucopyranoside-7-O-α-rhamnopyranoside (**9**) [24]*
Yellow amorphous power, R_f 0.43 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 257, 295sh, 358; (+NaOMe) 266, 407; (+$AlCl_3$) 273, 301sh, 339sh, 426; (+$AlCl_3$/HC1) 269, 298sh, 358, 402; (+NaOAc) 259, 396sh, 367, 415; (+NaOAc/H_3BO_3) 261, 295sh, 378. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 7.6 (2H, m, H-2′,6′); 6.82 (1H, d, *J*= 8.5, H-5′); 6.77 (1H, d, *J*=2.0, H-8); 6.48 (1H, d, *J*= 2.0, H-6); 5.55 (1H, d, *J*=2.0, H-1‴); 5.48 (1H, d, *J*= 7.5, H-1″); 3-4 (m, sugar protons overlapped with -OH proton signals); 1.12 (3H, d, *J* = 6.0, H-6‴). ESI-MS: *m/z* 609 $[M-H]^-$.

*Quercetin 3-O-α-rhamnopyranoside-7-O-β-glucopyranoside (**10**) [23]*.
Yellow amorphous power, R_f 0.46 (BAW). UV spectral data, λ_{max}(nm): (MeOH) 257,295sh, 356 (+NaOMe) 266, 405; (+$AlCl_3$) 273, 300sh, 340sh, 426; (+$AlCl_3$/HC1) 269, 299sh, 358, 401; (+NaOAc) 259, 396sh, 367,416; (+NaOAc/H_3BO_3) 261, 295sh, 375. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 7.55 (2H, m, H-2′,6′); 6.87 (1H, d, *J*= 8.5, H-5′); 6.8 (1H, d, *J*=2.0, H-8); 6.45 (1H, d, *J*= 2.0, H-6); 5.42 (1H, d, *J*=2.0, H-1″); 5.01 (1H, d, *J*= 7.5, H-1‴); 3-4 (m, sugar protons overlapped with -OH proton signals); 0.88 (3H, d, *J*= 6.0, H-6″). ESI-MS: *m/z* 609 $[M-H]^-$

Plant material

The plant material was collected from Giza field and identified by Dr. Mona Mohamed Marzouk on March 2007. A voucher specimen (M 820) was deposited in the herbarium of the National Research Center (CAIRC).

Extraction and isolation

The air-dried powdered aerial part of *R. raphanistrum* (1.2 Kg) was extracted with 70% EtOH (3×3L). The solvent was evaporated under reduced pressure at 50ºC. The dried 70% aqueous ethanol extract was defatted with petroleum ether. The defatted aqueous ethanol extract (245 g) was then subjected to Polyamide CC (5.5×125cm) eluted with H_2O and followed by H_2O/MeOH mixtures of decreasing polarity. A total of 49 fractions were collected, each 250 mL; these were controlled by PC to give four main fractions (RA-RD). RA was chromatographed on Sephadex column using H_2O:MeOH (1:1) for elution yielding two sub fractions, each was subjected to PPC using BAW and followed by Sephadex column using methanol to yield compounds **6** (48 mg) and **7** (64 mg). CC of RB on polyamide column (2.5×45cm), gradient elution with H_2O/MeOH, gave two major sub fractions; RB1 and RB2. Repeated CC for each on Sephadex using MeOH: H_2O (1:1), followed by PPC using BAW then 15% HOAc, afforded compounds **4** (36 mg), **8** (47 mg), **5** (34 mg) and **10** (30 mg), respectively. RC was applied on PPC using BAW then H_2O double solvent yielded compounds **9** (48 mg) and **3** (14 mg). Compound **1** (11 mg) and **2** (18 mg) were obtained from RD by PPC using 15% HOAc and followed by BAW two times.

LC-ESI-MS analysis

LC-ESI-MS analysis system consists of HPLC (Waters Alliance 2695) and mass spectrometry (Waters 3100). The mobile phases were prepared daily by filtering through 0.45 μm membrane disc filter and degassed by sonication before use. The mobile phase for gradient elution consists of two solvents: solvent A (0.1% formic acid (FA) in H_2O) and solvent B (0.1% FA in CH_3CN/MeOH (1:1; *v*/*v*). The linear gradient profile was as follows: 95% A (5 min), 95-90% A (10 min), 90-50% A (55 min), 50-95% A (65 min), and 95% A (70 min). The injection volume was 10 μL. The flow rate (0.6 mL/min) was split 1:1 before the MS interface. The negative ion mode parameters were as follows: source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 50 L/h, cone voltage 50 eV, capillary voltage 3 kV, and desolvation gas flow 600 L/h. Spectra were recorded in the ESI negative mode between 50-1000 *m/z*. The peaks and spectra were processed using the Maslynx 4.1 software. The flavonoids isolated in the present study together with other pure flavonoids obtained from phytochemical and plant systematic department were used as authentics. Known peaks were identified by comparing their retention time and mass spectrum with the flavonoids isolated in the present study. Unknown peaks were tentatively identified by comparing their mass fragmentation pattern with literatures.

Cell culture and sample treatment

The cell line under investigation was human breast adenocarcinoma (MCF7), human hepatocellular carcinoma cell line (HEPG2), human lung carcinoma (A549) and human colon cell line (HCT116). They were purchased from American Tissue Culture Collection. HEPG2, MCF7 and HCT116 cells lines were cultured in RPMI 1640 medium while A549 cell line was cultured in DMEM media. Media are supplemented with 1% antibiotic antimycotic mixture (10,000 UmL^{-1} potassium penicillin, 10,000 μgmL^{-1} streptomycin sulphate and 25 μgm^{-1} amphotericin B), 1% L-glutamine and 10% fetal bovine serum. According to the cells growth profile, cells were seeded with a density of 1×10^4 cell per well. This number was sufficient to give a reliable reading with the MTT assay, which corresponded well with the cell number and was the one that gave exponential growth throughout the incubation period with the tested sample [25].

RESULTS AND DISCUSSION

Identification of the isolated flavonoids

The present work deals with the isolation of ten flavonoids on the bases of chromatographic techniques [19] (Fig.**1**).

Their structure clarification was carried out through color reactions, R_f values, chemical investigations (mild and complete acid hydrolysis) and physical investigations (MS, UV and NMR) [19, 20-23, 26]. Further authentication was carried out by comparison of their spectroscopic data with previously published values. They were identified as kaempferol (**1**), kaempferol 3-*O*-α-arabinopyranoside (**2**), kaempferol 3-*O*-α- rhamnopyranoside (**3**), kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside (**4**), kaempferol 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside (**5**), kaempferol 3,7-di-*O*-α-rhamnopyranoside (**6**), kaempferol 3-*O*-(2″-β-glucopyranosyl)-α-rhamnopyranoside-7-*O*-α-rhamnopyranoside (**7**), quercetin 3-*O*-α-rhamnopyranoside-7-*O*-α-arabinopyranoside (**8**), quercetin 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside (**9**), quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside (**10**).

Table 1. Tentative identification of phenolic compounds in *R. raphanistrum*

Peak no.	t_R (min)	M	[M – H]$^-$	*m/z* fragments	Tentative identification
1	28.30	486	485	313,167, 97	Unknown
2	31.06	610	609	463, 447, 301	Quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-glucopyranoside (**10**)*
3	31.64	610	609	463, 447, 301	Quercetin 3-*O*- β -glucopyranoside-7-*O*-α-rhamnopyranoside (**9**)*
4	32.23	580	579	447, 285	Kaempferol 3-*O*- pentosyl hexoside #
5	32.56	580	579	447, 433, 301	Quercetin 3-*O*- β -rhamnopyranoside-7-*O*- β -arabinopyranoside (**8**)*
6	33.15	594	593	447, 431, 285	Kaempferol 3-*O*- β -glucopyranoside-7-*O*-α-rhamnopyranoside (**4**)*
7	33.81	756	755	609, 447, 301	Quercetin 3-*O*- glucosyl rhamnoside -7-*O*-rhamnoside #
8	34.40	594	593	447, 285	Kaempferol 3-*O*-α- rhamnopyranoside-7-*O*- β -glucopyranoside (**5**)*
9	35.8	564	563	417, 285	Kaempferol3-*O*-pentose-7-*O*- rhamnose #
10	35.90	740	739	593, 431, 285,	Kaempferol 3-*O*-(2´´- β –glucopyranosyl)-α-rhamno pyranoside-7-*O*-α-rhamnopyranoside (**7**)*
11	37.40	578	577	431, 285	Kaempferol 3,7-di-*O*-α-rhamnopyranoside (**6**)*
12	38.41	902	901	755, 470, 431, 285	Kaempferol -3- dihexosyl-rhamnoside7- rhamnoside #
13	39.32	418	417	285	Kaempferol 3-*O*- β -arabinopyranoside (**2**)*
14	42.11	432	431	285	Kaempferol 3-*O*-α-rhamnopyranoside (**3**)*
15	43.58	448	447	301	Quercetin 7-*O*-α-rhamnopyranoside †
16	46.51	286	285	151, 96, 62	Kaempferol (**1**)*
17	48.51	432	431	285	Kaempferol 7-*O*-α-rhamnopyranoside †

** Compounds isolated in the present study.*
†Compound isolated previously from the plant and identified by comparing their mass spectrum with literatures [17].
 # Compounds detected for the first time by LC-ESI-MS technique.

The plant extract was also applied to LC-ESI-MS analysis which is an ideal solution to detect the compounds that present in minute amounts and could not be identified clearly due to their minority. The ten isolated flavonoids were used as authentic samples and were observed in the chromatogram as peaks **2**, **3**, **5**, **6**, **8**, **10**, **11**, **13**, **14** and **16** (Fig. **2**, Table **1**). Other additional peaks were also observed and represented as peaks **1**, **4**, **7**, **9**, **12**, **15** and **17**.

Peak **4** (t_R= 32.23 min) showed a molecular ion peak at *m/z* 579. The MS fragments at *m/z* 447 (loss of 132) and 285 (loss of 162), suggesting that it might be a kaempferol diglycoside containing pentose and hexose moieties. No MS fragment was detected at *m/z* 417 which established that both sugar moieties were linked to the same phenolic hydroxyl as a disaccharide residue [27]. Moreover, the appearance of *m/z* 447 (loss of pentose) then at 284 (loss of hexose) could be suggested that the pentose moiety is in a terminal position, while the hexose one is directly attached to the aglycone (pentosyl hexoside) [28]. Therefore, peak **4** was tentatively identified as kaempferol 3-*O*-pentosyl hexoside.

Another kaempferol derivative; peak **9** (t_R= 35.8 min), showed a molecular ion peak at *m/z* 563. The appearance of two monoglycoside analogues at *m/z* 431 [M-H]$^-$ (loss of 132) and *m/z* 417 [M-H]$^-$ (loss of 146), confirming the substitution with rhamnose and pentose units through two different OH groups of kaempferol aglycone [29]. The fragment at *m/z* 431[kaempferol+rhamnose-H]$^-$ has a very low relative intensity than of *m/z* 417 [kaempferol+pentose-H]$^-$, indicating 3-pentoside and 7-rhamnoside substitution [30]. Peak **9** was therefore putatively identified as kaempferol 3-*O*-pentoside-7-*O*-rhamnoside.

Peak **7** (t_R= 33.8 min) showed a molecular ion peak at *m/z* 755. The MS fragments at *m/z* 609 (loss of 146), 447 (loss of 162) and 302 (loss of 146), suggesting that it may be quercetin glycosides containing two rhamnose and one hexose moieties. The loss of rhamnose unit (-146) at first suggested its substitution at position 7, confirmed by previous studies which showed that first fragmentation of flavonol compound is always due to the breakdown of *O*-glycosidic bond at position 7, followed by breakdown of molecule linked to position 3 [31]. The appearance of *m/z* 447 (loss of 162) then at 302 (loss of 146), portentous the presence of a disaccharide residue at 3 OH group [31]; the hexose moiety is in a terminal position, while the rhamnose one is directly attached to the aglycone [28]. Thus peak **7** is tentatively assigned as quercetin 3-*O*-hexosyl-rhamnoside-7-*O*-rhamnoside, which might be the quercetin analogue of compound **7** (kaempferol 3-*O*-(2′′-β-glucopyranosyl)-α-rhamnopyranoside-7-O-α-rhamnopyranoside).

Only a tetra-glycoside flavonoid derivative was tentatively assigned for peak **12** in the chromatogram (t_R= 38.41 min) and considered to be the first reported in radish plant. It exhibited an [M-H]$^-$ ion at *m/z* 901 (major peak) and produced a fragment at *m/z* 755 corresponding to the firstly loss of a rhamnose moiety (-146amu,rhamnose) at 7-OH group of kaempferol aglycone [31]. The appearance of fragment ion at *m/z* 469 which revealed a trisaccharide residue composed of two hexose and one rhamnose molecules linked with position 3 of kaempferol aglycone at *m/z* 285. The chromatogram also showed another fragment at *m/z* 431 which indicated that the rhamnose is the directly attached sugar of the trisaccharide residue [28]. These results allowed the tentatively identification of peak **12** as kaempferol-3-dihexosyl-rhamnoside-7-rhamnoside.

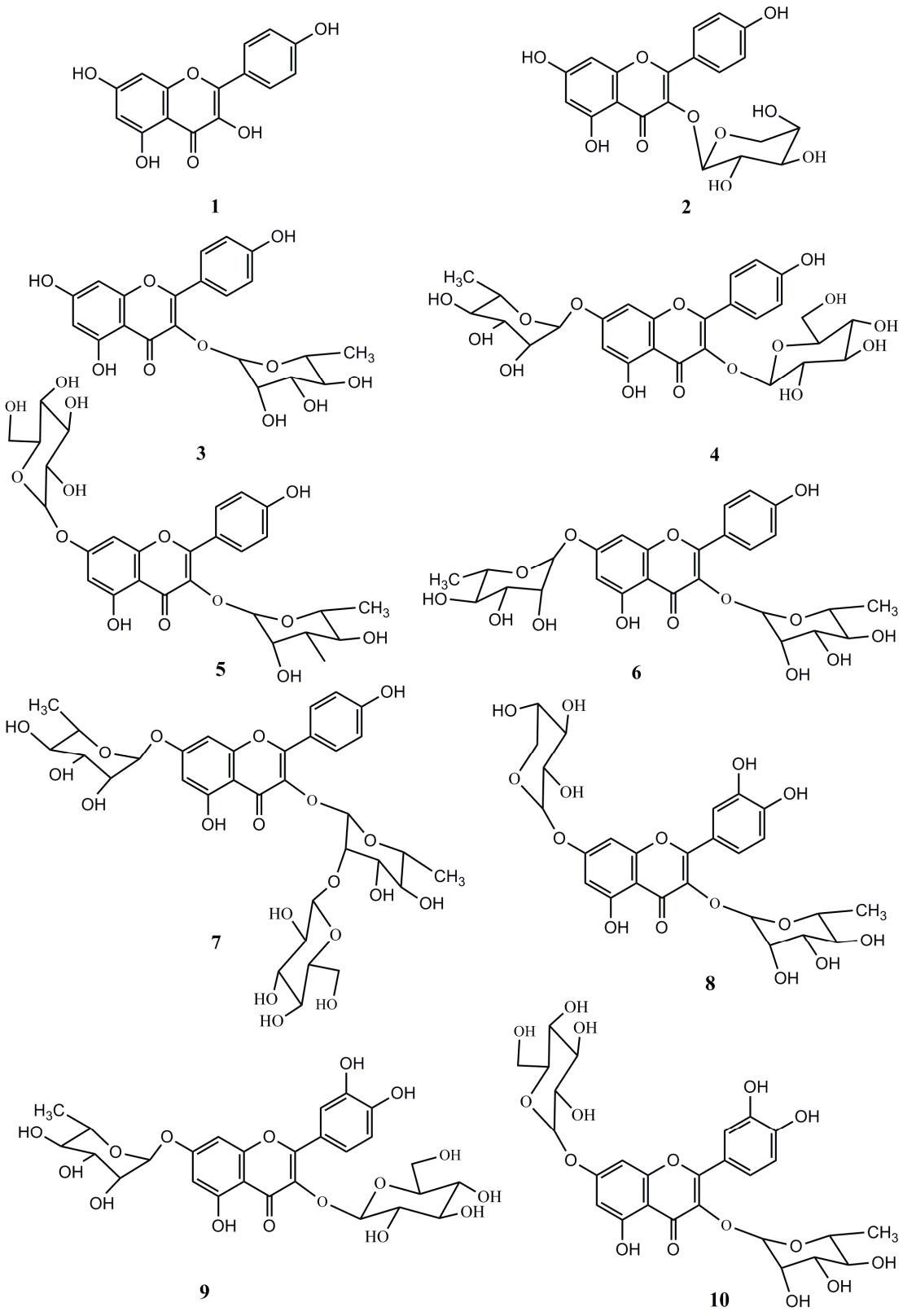

Fig. 1: Chemical structures of the isolated flavonoids from *R raphanistrum*

Peaks **15** & **17** at t_R 43.58 & 48.51, respectively, were associated to flavonol group. The proposed candidate for peak **15** was quercetin derivative which show molecular ion at *m/z* 447 $[M-H]^-$ and a fragment at *m/z* 301 $[quercetin-H]^-$, while the fragment at m/z 285 $[kaempferol-H]^-$ was observed in peak **17**, indicating a kaempferol derivative; *m/z* 431 $[M-H]^-$. Both peaks showed the loss of rhamnose moiety (-146 mu, rhamnose), suggesting their tentative assigning

as quercetin 7-*O*- rhamnopyranoside and kaempferol 7-*O*-rhamnopyranoside, respectively. Both compounds were previously reported from the same plant [17].

In vitro cytotoxic activity

The results indicated that *R. raphanistrum* extract showed moderate activity against HEPG2 and MCF7 at 100 mg/mL with cell viability of 62.4% and 57.9%, respectively as well as weak effect against HCT116 and A549 with cell viability of 37.8% and 12.6%, respectively.

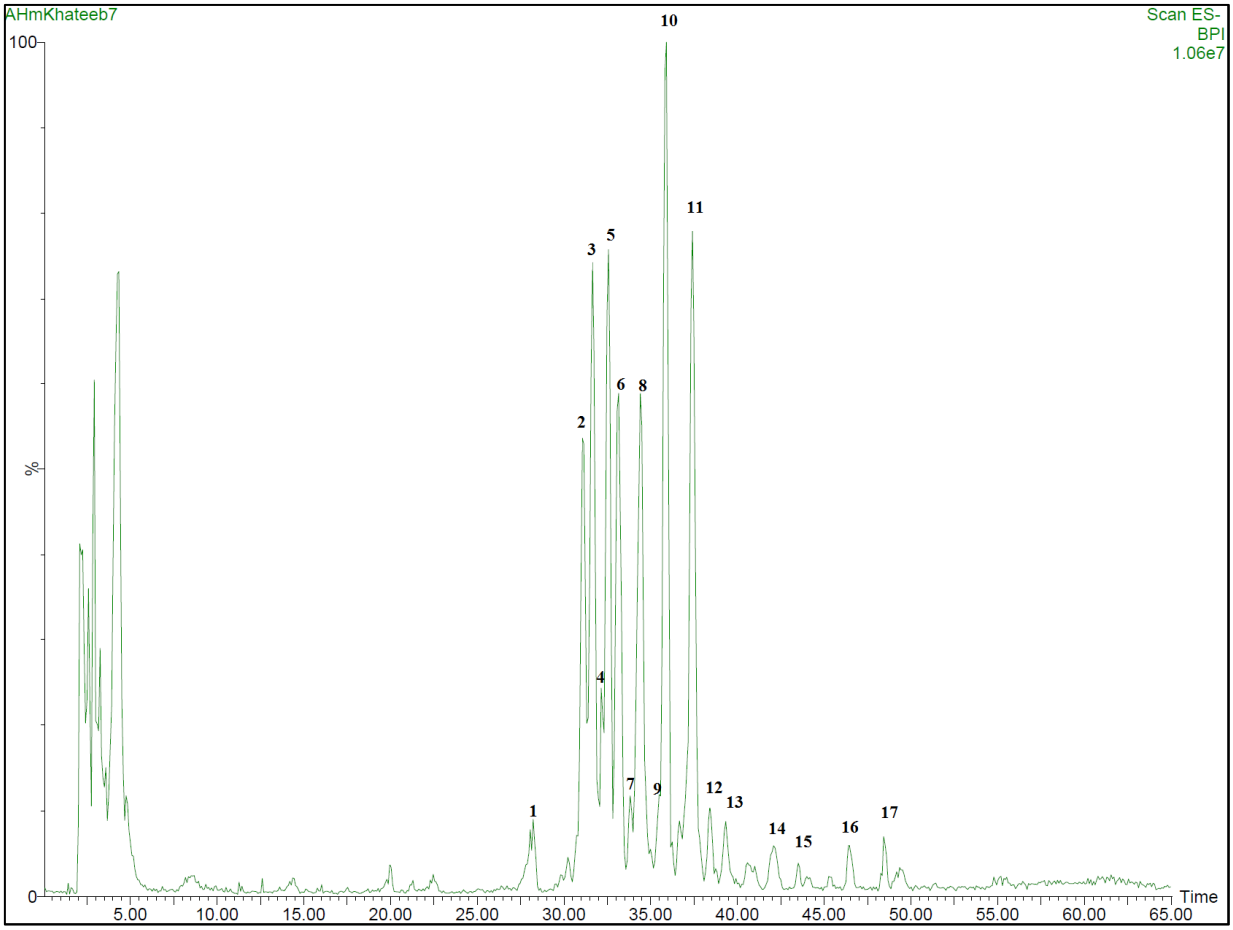

Fig. 2. LC-ESI-MS chromatogram of flavonoids in *R raphanistrum* aerial plant extract; 1: Unknown, 2: quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside 3: quercetin 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside, 4: kaempferol 3-*O*- pentosyl hexoside, 5: quercetin 3-*O*- α -rhamnopyranoside-7-*O*-α-arabinopyranoside, 6: kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside, 7: quercetin 3-*O*- glucosyl rhamnoside-7-*O*-rhamnoside 8: kaempferol 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside, 9: Kaempferol3-*O*-pentose-7-*O*- rhamnose, 10: kaempferol 3-*O*-(2″- β-glucopyranosyl)- α- rhamnopyranoside-7-*O*-α-rhamnopyranoside, 11: kaempferol 3,7-di-*O*-α-rhamnopyranoside, 12: kaempferol -3- dihexosyl-rhamnoside7-rhamnoside, 13: kaempferol 3-*O*-α-arabinopyranoside, 14: kaempferol 3-*O*-α- rhamnopyranoside, 15: quercetin 7-*O*-α- rhamnopyranoside, 16: kaempferol, 17: kaempferol 7-*O*-α- rhamnopyranoside

CONCLUSION

Ten flavonoids were isolated and identified from *R. raphanistrum* extract, among them 8 compounds were isolated for the first time. LC-ESI-MS analysis was an ideal solution for the tentative identification of the minor compounds. Kaempferol and quercetin nuclei with high grade of glycosylation were identified for the first time from the plant under investigation. *R. raphanistrum* extract showed moderate cytotoxic activity, against HEPG2 and MCF7 at 100 mg/mL.

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