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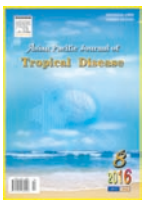

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Phenolic profiling of *Rorippa palustris* (L.) Besser (Brassicaceae) by LC-ESI-MS: Chemosystematic significance and cytotoxic activity

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ABSTRACT

Objective: To investigate the phenolic profile of *Rorippa palustris* (*R. palustris*) compared to Egyptian *Rorippa* species to evaluate their chemosystematic significance as well as screen cytotoxicity of *R. palustris* extract.

Methods: The chemical components of defatted hydroalcoholic *R. palustris* extract were analyzed using liquid chromatography-electrospray ionization mass spectrometry technique. The cytotoxic activity was evaluated by using MTT assay against four carcinoma cell lines.

Results: Sixteen compounds corresponding to flavonoids (kaempferol and quercetin derivatives), phenolic acids (gallic, coumaric and ferulic acids derivatives) and an anthocyanin (delphinine derivative) were identified or tentatively characterized, of which ten compounds were detected for the first time from *R. palustris*. Moreover, *R. palustris* extract showed a moderate activity against MCF7 and A549 cell lines at 100 mg/mL with cell viability of 47.3% and 65.4%, respectively.

Conclusions: The phenolic profile and the morphological characters of *R. paluatris* seem to be in relation with those of *Rorippa indica*, which can be considered as an indicative parameter for its medicinal importance.

1. Introduction

The genus *Rorippa* Scop. is one of the largest genera in the tribe Arabideae of family Brassicaceae, comprising approximately 80 species distributed worldwide except Antarctica[1,2]. It includes an important Chinese traditional herb *Rorippa indica* (L.) Hiern (*R. indica*), which is used as animal forage and treatment for fever, cough, rheumatism and inflammation[3]. In Egypt, the genus *Rorippa* is represented by three wild species: *R. indica*, *Rorippa palustris* (L.) Besser (*R. palustris*) and *Rorippa integrifolia* Boulos[4]. *R. palustris*, a species closely morphologically related to *R. indica*, is the widely distributed species of the genus, commonly named as bog yellowcress, yellow watercress and marsh yellowcress[5].

The chemical constituents of *Rorippa* species were found to contain phenolic acids, flavonoids, glucosinolates and isothiocyanate[6-10]. Some *Rorippa* species possess a highly antioxidant activities[11] and cytotoxicity against human MDA-MB-231 breast cancer cells[12]. Also, they have the ability to reduce lymphocyte DNA damage which could be related to a reduced risk of cancer[13]. In the present study, *R. palustris* was selected for further phytochemical investigation using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) to evaluate its chemosystematic importance in relation to related taxa of Egypt.

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Additionally, the cytotoxic activity of its extract against breast (MCF7), hepatocellular (HEPG 2), lung (A549) and colon (HCT116) carcinoma cell lines was investigated.

2. Materials and methods

2.1. Plant material

R. palustris was collected and identified by Dr. Mona M. Marzouk from Bin El-Bahrin Island, near Pharos garden, Giza, in March 2009. A voucher specimen (No. M42) was deposited in the herbarium of the National Research Center (CAIRC).

2.2. Extraction

The air-dried powdered aerial parts of *R. palustris* were extracted three times with 70% MeOH. The solvent was evaporated under reduced pressure at 50 ˚C. The dried hydroalcoholic extract was defatted with petroleum ether[14].

2.3. Acid hydrolysis and paper chromatography

A total of 100 mg of *R. palustris* extract (RPE) was hydrolyzed with 10 mL hydrochloric acid (2 mol/L) at 100 ˚C for 2 h. The acidic solution was fractioned with 10 mL ethyl acetate after cooling. Ethyl acetate layer was dried with anhydrous Na_2SO_4 then evaporated. The hydrolyzed extract was subjected to one dimension paper chromatography (PC) Whatman No. 1 (Whatman Ltd., Maidstone, Kent, England) using solvent systems; 50% AcOH (H_2O: AcOH, 1:1) and BAW (*n*-BuOH–AcOH–H_2O 4:1:5, upper layer) to detect the aglycones. Also the aqueous layer was carefully neutralized, then subjected to PC investigation using BBPW (benzene: *n*-BuOH: pyridine: H_2O; 1:5:3:3, upper layer) to detect the sugars[14]. Flavonoid aglycones (Fluka AG, Bucns SG, Switzerland) and sugar samples (E. Merck, Darmstadt, Germany) were used as authentic references.

2.4. LC-ESI-MS analysis of RPE

LC-ESI-MS analysis system consists of high performance liquid chromatography (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 in H_2O) and solvent B (0.1% formic acid 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 50–1 000 *m/z*. The peaks and spectra were processed using the Maslynx 4.1 software[15]. Known peaks were identified by comparing their retention time and mass spectrum with the flavonoid standards (95% purity; UV, nuclear magnetic resonance) which obtained from this research group (Phytochemical and Plant Systematic Department, NRC)[10,16-21]. Other peaks were tentatively identified by comparing the mass spectrum with literatures.

2.5. Cell culture and sample treatment

The investigated human carcinoma cell lines were breast (MCF7), hepatocellular (HepG2), lung (A549) and colon (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 Dulbecco's modified Eagle's medium media. Media are supplemented with 1% antibiotic antimycotic mixture (10 000 IU/mL potassium penicillin, 10 000 μg/mL streptomycin sulphate and 25 μg/mL 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[20].

3. Results

3.1. Acid hydrolysis

The PC of the ethyl acetate extract gave two spots which have R_f and color reaction similar to kaempferol and quercetin aglycones. Galactose, glucose, arabinose and rhamnose were detected as sugar moieties in the aqueous extract. Complete hydrolyzing indicated that all glycoside were in *O*-glycoside form.

3.2. Identification of phenolics using LC-ESI-MS analysis

Nineteen peaks were detected in RPE chromatogram (Table 1, Figure 1). The flavonoid contents were identified for peaks 1, 6–10, 13, 15, 17 and 18. The peaks 1, 9, 10, 17 and 18 were predicted to be kaempferol derivatives, while the others were related to quercetin aglycone.

Peaks 1, 9 and 10 presented the same molecular ion peak at *m/z* 593 provided the presence of kaempferol and a disaccharide residue (rhamnose and hexose). The hexose moiety was confirmed to be glucose or galactose by direct comparison with standards in the acidic hydrolyzed extract.

The flavonoid 1 (Rt = 19.90 min) showed fragments of *m/z* 447 (by loss of 146) and *m/z* 285 (loss of 162), suggesting that the rhamnose moiety was in a terminal position in the disaccharide residue, while the hexose one was directly attached to the aglycone (*i.e.* rhamnosyl-hexoside)[22]. It was further identified as kaempferol 3-*O*-β-(2"-*O*-α-rhamnopyranoside)-glucopyranoside by direct comparison with an authentic standard and previously isolated from *R. palustris*[10].

Another kaempferol isomers 9 (Rt = 33.20 min) and 10 (Rt = 34.48

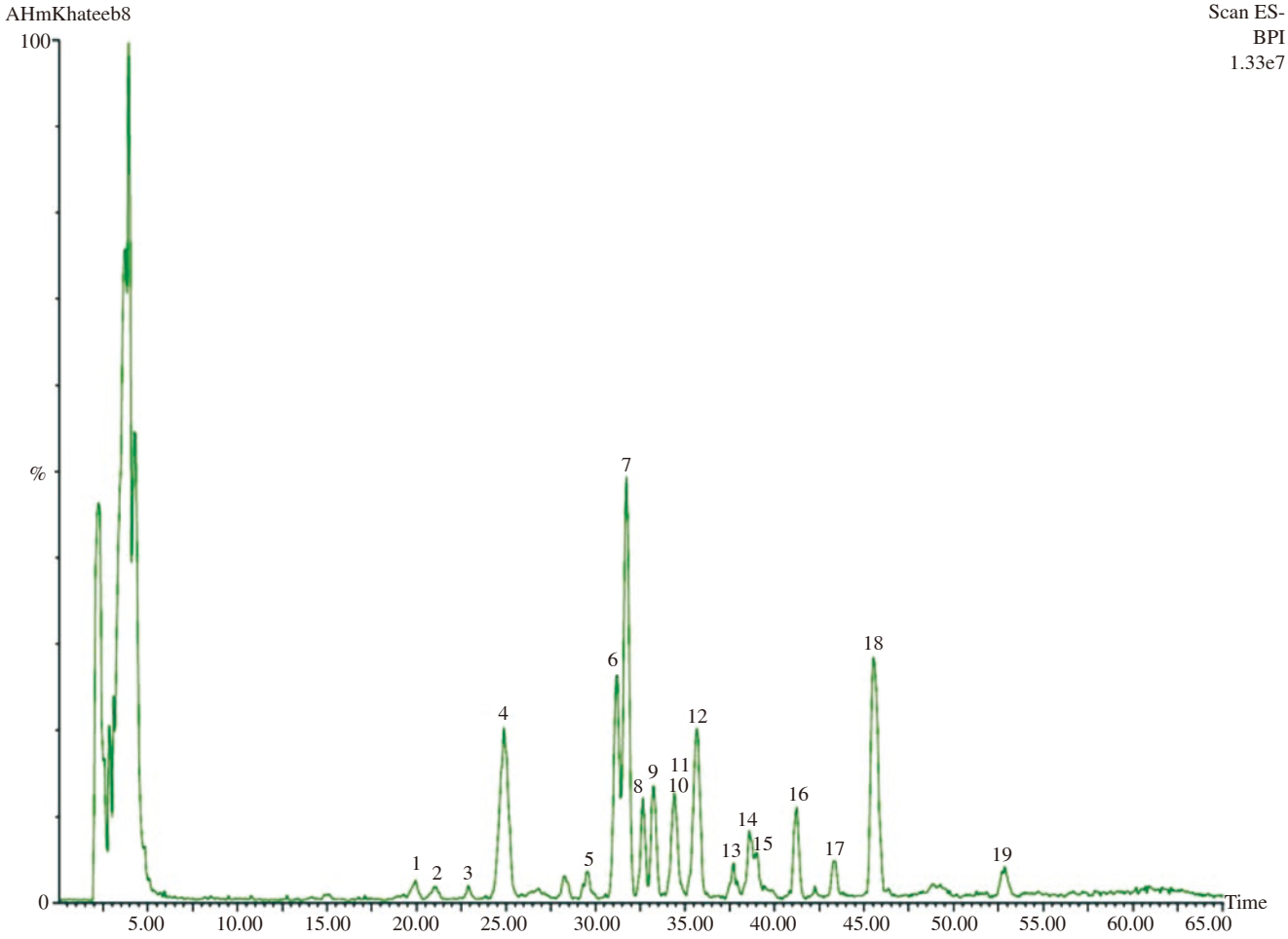

Figure 1. LC-ESI-MS chromatogram of phenolic compounds in RPE.

Table 1

Tentative identification of phenolic compounds in RPE using LC-ESI-MS technique.

Peak No.	Rt (min)	M	[M –H]-	*m/z* fragments	Identification
1	19.90	594	593	447, 285	Kaempferol 3-*O*- (2''-*O*-α- rhamnopyranoside)- β-glucopyranoside[a]
2	21.04	683	682	475, 93	Unknown
3	22.80	286	285	153, 109	Dihydroxybenzoylpentose[b]
4	24.88	477	476	169, 125	Gallic acid derivative
5	29.55	194	193	167, 108, 61	Unknown
6	31.22	610	609	463, 447, 301	Quercetin 3-*O*-β-galactopyranoside 7-*O*-α-rhamnopyranoside[a]
7	31.81	610	609	463, 447, 301	Quercetin 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside[a]
8	32.65	580	579	447, 431, 301	quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-arabinopyranoside[c]
9	33.20	594	593	447, 431, 285	Kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside[a]
10	34.48	594	593	447, 431, 285	Kaempferol 3-*O*-α- rhamnopyranoside-7-*O*-β-glucopyranoside[c]
11	34.48	280	279	163, 133, 116	Coumaroyl malate[b]
12	35.60	309	308	193, 134, 116	Feruloyl malate[b]
13	37.10	464	463	301	Quercetin 3-*O*-β-glucopyranoside[c]
14	38.58	678	677	193, 161, 134	Ferulic acid trihexoside
15	39.11	742	741	609, 447, 301	Quercetin 3-glycosyl-rhamnoside-7-arabinoside
16	41.25	790	789	465, 303	Delphinine trihexoside[b]
17	43.34	448	447	285	Kaempferol 3-*O*-β-glucopyranoside[a]
18	45.51	448	447	285	Kaempferol 7-*O*-β-glucopyranoside[a]
19	52.86	148	147	117	Unknown

[a]: Compound isolated previously from *R. palustris* and confirmed by comparing their retention times and mass spectrum with the authentic; [b]: Compounds tentatively identified based on the mass spectral data cited in the literature; [c]: Compounds identified by comparing their retention times and mass spectrum with the authentic. Rt: Retention time.

min) showed fragments at *m/z* 447 $[M-H-146]^-$ (loss of rhamnose), 431 $[M-H-162]^-$ (loss of hexose), 285 $[M-H-308]^-$ (loss of rhamnose + hexose), indicating the presence of kaempferol, hexose and rhamnose. The appearance of two fragments (*m/z* 447, *m/z* 431), confirming the glycosylation of two OH groups at different positions of kaempferol aglycone[23]. Peak 9 was identified as kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside which was isolated previously from *R. palustris*[10] and *R. indica*[8], while peak

10 was identified as kaempferol 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside, detected for the first time from *R. palustris*. Their full characterization was confirmed by comparing the retention time and mass spectral data with the authentic samples.

Peaks 17 and 18 have the same molecular ion peak at *m/z* 447 and fragment at *m/z* 285 afforded the presence of kaempferol and one hexose moiety. Both peaks were compared with the Rt and mass fragmentation of the standards and identified to be kaempferol 3-*O*-β-glucopyranoside and kaempferol 7-*O*-β-glucopyranoside, respectively.

The quercetin derivatives 6 and 7 provided the largest peaks in the chromatogram of RPE. In comparison to the authentic reference, they were identified as quercetin 3-*O*-β-galactopyranoside 7-*O*-α-rhamnopyranoside and quercetin 3-*O*-β-glucopyranoside 7-*O*-α-rhamnopyranoside, respectively. Both compounds were isolated previously from the same species[10].

Peak 8 (Rt = 32.65 min) showed molecular ion peak $[M-H]^-$ at *m/z* 579, yielding fragments at *m/z* 447 (-132; pentose) and *m/z* 431 (-146; rhamnose), then a major fragment appeared after the loss of the two moieties at *m/z* 301 [quercetin-H]$^-$. The presence of two fragments of mono-glycosyl analogues was always due to the *O*-substitution at two different positions of the aglycone[23]. Peak 8 was further identified as quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-arabinopyranoside supported by the matching of mass fragmentation and Rt with the authentic sample. In the same way, peak 13 with *m/z* 463 $[M-H]^-$ ion was confirmed to be quercetin 3-*O*-β-glucopyranoside. Both compounds were detected for the first time from *R. palustris*.

Peak 15 (Rt = 39.11 min) was considered to be the only triglycoside flavonoid identified in RPE chromatogram with a molecular ion at *m/z* 741 $[M-H]^-$ and produced fragments of *m/z* 609 (loss of 132), *m/z* 447 (loss of 162) and *m/z* 301 (loss of 146), suggesting that it might be quercetin containing three glycosidic units (pentose, hexose and rhamnose). The pentose moiety was identified to be arabinose and confirmed by data obtained from the acid hydrolysis. The presence of fragments at *m/z* 609 (loss of arabinose) and *m/z* 447 (loss of hexose) then at *m/z* 301 (loss or rhamnose) may indicate the presence of a diglycosidic residue (hexosyl rhamnoside) and a monoglycosidic moiety (arabinoside) was attached to the quercetin aglycone through two different OH groups[22]. Therefore, peak 15 may be tentatively identified as quercetin 3-glycosyl-rhamnoside-7-arabinoside.

The phenolic acid derivatives were observed in peaks 4, 11, 12, and 14. Peak 4 was tentatively identified as gallic acid derivative in which the molecular ion was observed at *m/z* 476 and product ions at *m/z* 169 [gallic acid-H]$^-$ and 125 by the loss of CO_2 molecule[24]. Peaks 11 and 12 with the masses $[M-H]^-$ at *m/z* 279 and *m/z* 308 were identified as esters of malic acid with coumaric acid and ferulic acid, respectively. After the ester breakdown with the loss of malate residue $[M-H-116]^-$, the fragments at *m/z* 163 and *m/z* 193 were corresponding to coumaroyl and feruloyl moieties, which were also confirmed by another fragment at *m/z* 134 $[M-H-116-CH_3-CO_2]^-$. The mass spectral data of 11 and 12 match to those reported in other genera of Brassicaceae family and are identified as coumaroyl malate and feruloyl malate, respectively[25]. Ferulic acid trihexoside was tentatively identified for peak 14, proved by the presence of the base peak fragment at *m/z* 193 (feruloyl moiety[26]. Another phenolic derivative was detected as *m/z* 285 (peak 3) and tentatively identified as dihydroxybenzoylpentose[3]. One anthocyanin skeleton was characterized for peak 16 which showed a molecular ion peak at *m/z* 789 and fragmentation ions at *m/z* 465 (loss of 324, *i.e.* lost two hexose residues) and at *m/z* 303 (delphinine-H). By comparing its fragmentation pattern with those reported in the literature[27], peak 16 was tentatively identified as delphinine trihexoside.

3.3. In vitro cytotoxic activity

The results indicated that RPE showed moderate activity against MCF7 and A549 at 100 mg/mL with cell viability 47.3% and 65.4%, respectively. However, it exhibited no evident cytotoxicity against HCT116 and HepG2 cell lines.

4. Discussion

In the present study, the LC-ESI-MS analytical technique was established for rapid and further identification of flavonoid constituents of RPE using numerous authentic samples previously isolated and identified from various cruciferous species[10,7-19] in comparing with those reported from *R. indica*[3,8]. Flavonoids were the major structure types in RPE chromatogram (Table 1, Figure 1) and represented as ten flavonol-*O*-glycosides of kaempferol and quercetin aglycones. Their full characterization was confirmed by comparing the retention times and mass spectral data with the authentic samples and identified as kaempferol 3-*O*-(2''-O-α-rhamnopyranoside)-β-glucopyranoside (1), quercetin 3-*O*-β-galactopyranoside 7-*O*-α-rhamnopyranoside (6), quercetin 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside (7), quercetin 3-*O*-α-rhamnopyranoside-7-*O*-β-arabinopyranoside (8), kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside (9), kaempferol 3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside (10), quercetin 3-*O*-β-glucopyranoside (13), quercetin 3-glycosyl-rhamnoside-7-arabinoside (15), kaempferol 3-*O*-β-glucopyranoside (17) and kaempferol 7-*O*-β-glucopyranoside (18). Additionally, other phenolic derivatives were tentatively identified on the bases of mass spectral data cited in the literature and characterized as dihydroxy benzoyl pentose (3), gallic acid derivative (4), coumaroyl malate (11), feruloy malate (12), ferulic acid trihexoside (14) and delphinine trihexoside (16)[24-27]. Compounds 3, 4, 8, 10 and 11–16 were reported for the first time from *R. paluatris*.

From the chemosystematics point of view, the flavonoid constituents support the classification of *R. palustris* and *R. indica* as two different species. Both species contain flavonols, commonly represented as *O*-glycoside derivatives of kaempferol and quercetin aglycones[3,8,10]. This finding is underlying their close relationships, while the difference occurs by presence of the acylation pattern of kaempferol and quercetin diglycosides in *R. indica*[3,8]. The same species were also characterized by the presence of isorhamnetin di-*O*-glycosides[3]. The two species are not only chemically different, they are also distinguished by the size of fruit and number of seeds. Siliqua; 7–30 × 1–2 mm with 60–110 seeds is characterized for *R. indica*, while silicula; 2.5–10 × 1.5–3.5 mm with 20–90 seeds for *R. paluatris*[28].

Because there is no chemical data concerning *Rorippa integrifolia*, more efforts were needed to further our knowledge about its relation to the other species.

Conflict of interest statement

We declare that we have no conflict of interest.

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