TITLE

An approach to the phytochemical profiling of rocket (*Eruca sativa* (Mill.) Thell)

RUNNING TITLE

Identification of bioactive compounds in rocket (*Eruca sativa* (Mill.) Thell)

AUTHORS' NAMES

Myriam Villatoro-Pulido[a], Feliciano Priego-Capote[b], Beatriz Álvarez-Sánchez[b], Shikha Saha[c], Mark Philo[d], Sara Obregón-Cano[e], Antonio De Haro-Bailón[e], Rafael Font[f], Mercedes Del Río-Celestino[f*]

[a] IFAPA Centro-Alameda del Obispo, Department of Plant Breeding and Biotechnology, Córdoba, Spain.

[b] Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, Córdoba, Spain.

[c] Phytochemicals and Health Programme, Institute of Food Research, Norwich Research Park, NR4 7UA Norwich, United Kingdom.

[d] Metabolomics and Mass Spectrometry, Institute of Food Research, Norwich Research Park, NR4 7UA Norwich, United Kingdom.

[e] Department of Plant Breeding, Institute of Sustainable Agriculture (IAS-CSIC), Alameda del Obispo s/n, 14080 Córdoba, Spain.

[f] IFAPA Centro La Mojonera, Department of Plant Breeding and Biotechnology, La Mojonera, Almería, Spain.

*Corresponding Author: e-mail: mercedes.rio.celestino@juntadeandalucia.es

ABSTRACT

BACKGROUND: *Eruca sativa* (rocket) contains a wide range of compounds with nutraceutical and organoleptical properties. These research has been aimed at characterizing the nutraceutical interest of four rocket accessions by analysis of glucosinolates, isothiocyanates, phenolics, carotenoids and carbohydrates. Different methods based on chromatographic separation with ultraviolet absorbance or mass spectrometry detection were used.

RESULTS: The total content of glucosinolates ranged from 14.02 to 28.24 µmol g^{-1} of dry weight. Glucoraphanin represented up to 52% of the total glucosinolates in leaves of one accession. Accessions showed differences in the hydrolysis of glucoraphanin to the isothiocyanate sulforaphane. No correlation between these compounds was observed, which insisted differences in the myrosinase activity within accessions. Rocket leaves had variable phenolic profiles represented by quercetin-3-glucoside, rutin, myricetin, quercetin and ferulic and *p*-coumaric acids. A high variability was observed for the total carotenoids ranged from 16.2 to 275 µg g^{-1} with lutein as the main carotenoid. Glucose was the predominant sugar representing >70% of the total soluble carbohydrates.

CONCLUSIONS: Some accessions could be candidates for future breeding programs because of their pattern of beneficial compounds for human health. However, further research is essential to evaluate the biological activity of these accessions before designing functional food.

Keywords*:* rocket; glucosinolate; isothyocyanate; phenol; carotenoid; carbohydrate.

INTRODUCTION

Many studies associate a highly significant cancer risk reduction with increasing *Cruciferae* consumption.[1] The term "rocket" refers mainly to *Eruca* and *Diplotaxis* genera within the *Cruciferae*. *Eruca sativa* contains a wide range of health-promoting phytochemicals including isothiocyanates (one of the degradation products of glucosinolates), phenolic compounds and carotenoids.[2–8]

When glucosinolates are exposed to myrosinase (thioglucoside glucohydrolase) during tissue damage, glucose and an unstable intermediate are formed. This intermediate degrades to produce a sulfate ion, and a variety of products including isothiocyanates, nitriles and, to a lesser extent, thiocyanates, epithionitriles and oxazolidines. The relative proportion of these hydrolysis products depends on the plant species studied, on the glucosinolate itself (as side chain substitution), and reaction conditions like pH, metal ions or epithiospecifier protein.[9]

Phenolics are the most abundant antioxidants in human diet. Considerable evidence indicates that some of the protective effects of phenols on fruits and vegetables may be due to flavonoids.[10] Carotenoids constitute one of the more important classes of plant pigments. Their antioxidant behaviour depends on the concentration and localization in the target cells, tissues or cellular compartments, as well as on other factors.[11]

No data on alditols and saccharides in *Eruca sativa* have been published yet, despite their contribution to organoleptical properties. The present work is part of an ongoing breeding program to obtain varieties of rocket with potential health benefits. The material for this work has been acquired from different European gene banks. After a previous screening of the material for glucosinolates, four accessions, covering a wide range of these compounds, were selected for an investigation to

identify different bioactive compounds, apart from glucosinolates, such as isothiocyanates, phenolic compounds, carotenoids and carbohydrates. The profiles may provide evaluation parameters for breeding programs and before planning strategies to design functional foods for improving consumer's health.

MATERIAL AND METHODS

Plant material and greenhouse experiments

Accessions of rocket used in this study were named according to the total content of glucosinolates: Low Glucosinolate content from 14 to 20 µmol g^{-1} (LGC1 and LGC2), and High Glucosinolate content from 27 to 30 µmol g^{-1} (HGC1 and HGC2). These accessions are part of a germplasm collection located at the IFAPA-La Mojonera, Almería (Southern Spain). Seeds of *Eruca sativa* LGC1, LGC2, HGC1 and HGC2 were obtained from Tozer Seeds Ltd. (Cobham, Surrey, U.K.), Faculté des Sciences Agronomiques (Gembloux, Belgium, accession number 187), Dipartimento di Scienze Botaniche (Palermo, Italy, accession number 122) and Botanischer Garten der Universität Karlsruhe (Germany, accession number 195), respectively. Seeds were germinated in Petri dishes for 48 h at 25ºC. Pots were placed in a greenhouse in December 2008 under natural light at 27/18ºC (day/night) and a relative humidity of 50/70% (day/night). When the plants reached 8–12 cm, they were transferred to a field in Córdoba, Spain (37"51'42'N, 04'48'00'W; 220m asl). The experiment was designed as a randomized complete block consisting of three replicate 5 m rows for each accession.

Sample pre-treatment and storage

Leaves from 10 randomly selected plants per replicate were harvested eight weeks

after transplanting and on the same day. They were washed, weighed to assess their biomass, and placed in Ziploc-type freezer bags at –80ºC for post-harvest storage. Immediately before analysis, the samples were freeze-dryed.

Glucosinolate analysis by liquid chromatography with ultraviolet absorbance detection (HPLC–UV)

An aliquot of freeze-dried sample (100 mg) was heated at 75ºC for 15 min in 70:30 methanol–water (2.5 mL) and 10 mM sinigrin (200 µl) as an external standard (Fluka, Seelze, Germany) according to the ISO norm.[12] The extract was centrifuged (5 min, 5000 *g*) and the pellet re-extracted with 70:30 methanol-water (2 ml). An aliquot (1 ml) of the combined glucosinolate extracts was pipetted onto the top of an ion-exchange column containing Sephadex DEAE-A25 (1 ml, 40-125 µm bead size, 30000 Da exclusion limit). Desulfation was carried out by addition of purified sulfatase (type H-1 from *Helix pomatia*) (Sigma-Aldrich) solution (75 µl). Desulfated glucosinolates were eluted with Milli-Q (Millipore) ultrapure water (2.5 ml) and analyzed with a 600 HPLC instrument (Waters) equipped with a model 486 UV tunable absorbance detector (Waters) fixed at a wavelength of 229 nm. Separation was carried out using a Lichrospher 100 RP-18 in Lichrocart column (125 mm × 4 mm i.d., 5 µm particle size, Merck). A gradient method was programmed for analysis of desulfo-glucosinolates using water (mobile phase A) and acetonitrile (mobile phase B) as chromatographic phases at flow rate 0.4 ml min^{-1}. The initial chromatographic conditions were set at 2% mobile phase B and then, linearly varied to 25% mobile phase B in 35 min. After analysis, the system was equilibrated for 10 min. The chromatograms were compared to the desulfo-glucosinolate profiles provided by three certified reference materials recommended by the U.E. and ISO (CRMs 366, 190 and

367) and using a pure standard GLS for the glucosativin (Commission of the European Communities, report EUR 13339 EN, 1-75).[13]

Sulforaphane, iberine and sulforaphane nitrile determination by liquid chromatography and mass spectrometry detection (HPLC–MS)

Freeze-dried leaves (40 mg) were hydrolyzed in phosphate buffered saline (PBS) (1 mL), incubated at 37°C for 2 h, and then centrifuged (13,000 *g*, 30 min at 4ºC) to generate isothiocyanates from glucosinolates. The supernatant was directly analyzed using liquid chromatography with photometric and mass spectrometric detection with a diode array detector and a single quadrupole mass spectrometry detector. The chromatographic mobile phases were 0.1% formic acid (v/v) in deionized water (mobile phase A) and 0.1% formic acid (v/v) in acetonitrile (mobile phase B). The chromatographic column was a Phenomenex Luna C-18 (150 mm × 4.6 mm i.d., 3 µm particle size). The chromatographic gradient was linearly programmed from 0% to 30% mobile phase B in 30 min and, finally, re-equilibration to 0% B for 10 min. The flow rate was set at 0.3 ml min^{-1}.

The target analytes were monitored by LC–MS with positive ionization mode using selected ion monitoring (SIM). The targeted ions were m/z 178.0, m/z 164.3 and *m/z* 146.0 for sulforaphane, iberin and sulforaphane nitrile, respectively. The target analytes were identified based upon their retention times and quantified with calibration curves generated using standards (LKT Laboratories, Inc., USA) with linear regression coefficients >0.99.

Erucin determination by gas chromatography/mass spectrometry analysis (GC–

MS)

The solution of isothiocyanates in PBS (0.5 ml) was added to the same volume of dichloromethane (CH_3Cl_2) for extraction of erucin. The organic phase was isolated and centrifuged at 13,000 *g* for 30 min at 4°C. The erucin content was measured by gas chromatography–mass spectrometry (GC–MS) with identification based on comparison to GC retention time and mass spectrum provided by erucin standard (LKT Laboratories, Inc., USA). GC–MS analysis was performed using a Trace GC Ultra™ (Thermo Scientific) operated in SIM mode with positive ionization by electron impact (EI+). Separation was carried out using a ZB-5mS (Phenomenex®, Netherlands), 30 m × 0.25 mm × 0.25 µm capillary column. The injection volume was 1 µl in splitless mode with a splitless time of 45 s and injector temperature of 250°C. The oven temperature program was linear with a ramp from 40°C min to 250°C at 10°C min^{-1}. The source and transfer line temperatures were 200 and 250ºC, respectively. The ions monitored for erucin identification (ER) were *m/z* 146, 161, 61 and 115.

Determination of the total phenolic fraction

The concentration of total phenolic compounds was estimated by a modified version of the Folin–Ciocalteu method[14] using gallic acid as standard, for which a calibration curve was run with solutions of 50, 100, 200, 300, 400, 500 and 600 mg l^{-1} of this compound. A 0.06 ml aliquot of extract 1.58 ml of distilled water, 0.1 ml of Folin–Ciocalteu reagent and 0.3 ml of Na_2CO_3 (20% w/v) were mixed and heated at 50ºC for 5 min. After 30 min, the absorbance was measured at 765 nm against a blank similarly prepared, but containing 70:30 ethanol–water mixture (pH 3.2) instead of

extract. Sodium carbonate (Panreac), Folin–Ciocalteu reagent and gallic acid (both from Sigma–Aldrich) were used to determine the total phenol fraction. The absorbance was measured with a ThermoSpectronic UV–visible Spectrometer (Thermo Fisher Scientific, USA).

Analysis of the phenolic fraction by liquid chromatography tandem mass spectrometry (HPLC–MS/MS)

Freeze-dried shoots (200 mg) were agitated overnight in 30 ml of 70:30 ethanol–water mixture at pH 3.2 fixed with formic acid.[15] Prior to HPLC–MS analysis, 100 µl of extract was evaporated to dryness and reconstituted in 100 µl of initial mobile phase for injection of 10 µl in the chromatograph coupled to an Agilent 6410 triple quadrupole (QqQ) mass analyzer with an electrospray ionization (ESI) source. The data were processed using a MassHunter Workstation Software from Agilent for qualitative and quantitative analysis. An Inertsil ODS-2 C18 analytical column (4.0 mm i.d.× 250 mm; 5 µm particle size, GL Sciences Inc., Tokyo, Japan) was used for chromatographic separation. Separation of the phenolic compounds was performed in 71 min, being the mobile phases A and B 0.4% aqueous formic acid and 50:50 (v/v) acetonitrile–methanol, respectively. The flow rate and the column oven temperature were set at 1 ml min^{-1} and 35ºC, respectively. The chromatographic method was as follows: the initial mobile phase was set at 4% of B, which was increased to 50% in 40 min and then to 60% B in 5 min. Finally, it was gradually changed to 100% mobile phase B in 3 min and maintained for 17 min. A re-equilibration step of 6 min was programmed after each chromatographic run. Analyses were carried out in selected reaction monitoring (SRM) negative ionization mode with nitrogen as drying and

nebulizing gas. The operating conditions of the ESI–QqQ, were: flow rate and temperature of drying gas 10 ml min^{-1} and 325ºC, respectively, nebulizer pressure 40 psi, capillary voltage 2700 V and dwell time 200 ms. The quantification transition and fragmentation conditions for each phenolic compound are shown in Supplementary Table 1.

The panel of phenolic compounds was composed of benzoic acid derivatives (protocatechuic, vanillic and syringic acids), methyl and ethyl esters from gallic acid, cinnamic acids (*p*-coumaric, ferulic and caffeic acids), stilbene (*trans*-resveratrol), the flavonols (kaempferol-3-O-rutinoside, quercetin, quercetin 3-ß-D-glucoside and myricetin) and the flavonol glycoside rutin hydrate, which were purchased from Sigma–Aldrich (St. Louis, MO, USA). The flavanols [(+)-catechin, (−)-epicatechin and procyanidins B1, B2 and A2] were from Extrasynthese (Genay Cedex, France).

Analysis of the carotenoid content by liquid chromatography with ultraviolet absorbance detection (HPLC–UV)

Carotenoids were extracted using a modification of the method described by Tadmor *et al.*[16] 400 mg of sample were rehydrated with 5 ml ethanol containing 1 mg ml^{-1} butylated hydroxytoluene (BHT) using a Polytron homogenizer. One ml of a 40% (w/v) KOH methanolic solution was added to each tube, and the samples were saponified for 10 min at 85ºC, cooled in an ice bath, then 2 ml of ice-cold water was added. The suspensions were extracted twice with 2 ml of hexane by vigorous vortexing followed by a 2000 *g* centrifugation for 10 min at room temperature. The upper hexane layers were pooled and evaporated to dryness and resuspended. The carotenoids were dissolved before injection in 800 µl of an acetonitrile–methanol–dichloromethane (45:20:35 v/v) solution, filtered through a 0.22 µm PTFE syringe

filter (Millipore) directly to sample vials, and 10 µl were injected into the chromatograph. The analyses were carried out with a Waters Symmetry C18 column (4.6 mm × 150 mm, 5 µm particle size) and a dual λ absorbance detector (model 2487).

The initial mobile phase consisted of acetonitrile–methanol (97:3, v/v) containing 0.05% (v/v) triethylamine. A linear gradient of dichloromethane from 0 to 10% in 20 min at the expense of acetonitrile was used, and then, the dichloromethane was kept constant at 10% until run completion. The flow rate was 1.0 ml min^{-1} and the column temperature was 30ºC. An absorbance detector was used to detect coloured carotenoids at 450 nm. The compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV-visible spectra with authentic standards purchased in Sigma-Aldrich.

Quantification was carried out by external standardization. Full standard curves were made in triplicate with five different concentrations for each carotenoid. The curves, which passed through or very near to the origin, were linear and bracketed the concentrations expected in the samples.

Analysis of the sugar fraction by gas chromatography with mass spectrometry detection

Two hundred mg of freeze-dried shoots was extracted by overnight agitation in 30 mL of 70:30 ethanol–water mixture at pH 3.2 fixed with formic acid as in the determination of the phenolic fraction.[15] A 150 µl aliquot of this extract was evaporated to dryness and reconstituted in 150 µl of derivatization solution, which consisted of 50-µl pyridine from Merck (Darmstadt, Germany), 98-µl *N*,*O*–Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 2 µl trimethylchlorosilane

(TMCS) from Sigma–Aldrich. The reaction mixture was vortexed at room temperature for 1 h, before the GC–MS analysis with a Factor Four capillary column (VF-5ms 30 m × 0.25 mm, 0.25 μm) from Varian (Palo Alto, USA). Thus, after derivatization, 1 μl of the analytical sample was injected into the chromatograph. The injector temperature was fixed at 280°C, and the injection was in the split/splitless mode. Helium at a constant flow-rate of 1.3 ml min^{-1} was used as carrier gas. The oven temperature program was as follows: initial temperature 65°C (held for 2 min), increased at 6°C min^{-1} to 300°C (held for 30 min). The ion-trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current 80 μA; transfer line, ion trap and manifold temperatures were kept at 280, 200 and 50°C, respectively. The MS/MS process was carried out by collision-induced dissociation (CID) in non-resonant excitation mode. Supplementary Table 2 shows the optimal MS/MS parameters for each compound. Carbohydrate standards D-(-)-arabinose, D-(+)-mannose, D-(-)-fructose, D-(-)-galactose, D-(+)-glucose, D-(+)-sucrose, and D-(+)-melazitose (the latter used as internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Glucosinolate content in rocket leaves

Thirteen glucosinolates (Fig. 1) were detected in rocket leaves belonging to the three chemical classes: seven aliphatic compounds (glucoerucin, gluraphanin, gluconapin, glucoiberverin, progoitrin, gluconapoleiferin, glucobrassicanapin and glucosativin), one aromatic (gluconasturtiin) and four indole compounds (4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, glucobrassicin and neoglucobrassicin). The GL profile

found in these four accessions was similar to the profiles reported by other authors in rocket leaves.[9, 17, 18]

The LGC1, LGC2, HGC1 and HGC2 accessions showed total GL content of 14.02, 19.4, 28.24 and 27.65 µmol g^{-1}, respectively (Table 1). In this work, the most abundant glucosinolates (i.e. glucoraphanin, glucosativin or glucoerucin) were identified in the four accessions ranging from 3.64 to 12.64 µmol g^{-1} dry weight (dw) for glucoraphanin, from 0.14 to 4.03 µmol g^{-1} dw for glucosativin and from 8.10 to 11.40 µmol g^{-1} dw for glucoerucin (Table 1). The highest contents of total GL and glucoraphanin were observed in HGC1 and HGC2 accessions, while the highest content of glucoerucin was also found in the HGC2 accession (Table 1).

In comparison to previous studies, higher levels of total GL, glucoerucin and glucosativin have been reported in rocket leaves in previous studies (55.4, 32.0 and 31.3 µmol g^{-1} dw, respectively).[9, 17] However, the concentrations of glucoraphanin (6.1 µmol g^{-1} dw) and glucobrassicanapin (0.2 µmol g^{-1} dw) found in the current survey were higher than those detected in previous characterizations.

Isothyocianate content in rocket leaves

The isothiocyanates detected were 1-ITC-4-(methylsulfinyl)-butane (sulforaphane), sulforaphane-nitrile, 3-methylsulphinylpropyl-ITC (iberin) and 4-(methylthio)butyl-ITC (erucin) (Table 1). Sulforaphane is derived from glucoraphanin and erucin is obtained from hydrolysis of glucoerucin, but also through *in vivo* reduction of the ITC sulforaphane.[19] The *in vivo* inter conversion of these two isothiocyanates and their structural similarity has suggested a similar biological activity. Iberin is formed from glucoiberin, which was not detected in these accessions. Accessions showed differences in the hydrolysis of glucoraphanin and formation of sulforaphane ranging

from 4.12% (LGC1 accession) to 97.35% (LGC2 accession). Pearson's correlation of glucoraphanin and sulforaphane was not significant in leaves of rocket (–0.38, $P > 0.05$), which suggested differences in the hydrolysis activity of related enzymes within accessions.

Sulforaphane mean content ranged from 0.15 to 5.90 µmol g^{-1} dw (Table 1) and sulforaphane nitrile was found ranging from 0.15 (HGC1 accession) to 0.97 (LGC2 accession) µmol g^{-1} dw. This last compound has been found to be ineffective as an inducer of some detoxification enzymes. The selection of accessions with low levels of the epithiospecifier protein might provide higher conversion of sulforaphane than sulforaphane nitrile with improved potency as anticarcinogenic food. LGC2 accession showed the maximum level for iberin (1.55 µmol g^{-1} dw). Erucin could be quantified only in the LGC2 accession (0.01 µmol g^{-1} dw). Our data are in accordance to those reported by Melchini *et al.*,[19] who reported values of sulforaphane and erucin of 3.46 µmol g^{-1} and 0.05 µmol g^{-1} dw, respectively. Although it has been previously published that erucin is the major ITC in rocket leaves,[20] other authors have stated that the most abundant ITC in rocket is sativin.[2]

Phenolic compounds in rocket leaves

Quercetin derived compounds such as quercetin-3-β-glucoside (isoquercetin) or rutin (quercetin-3-O-rutinoside) were the most abundant flavonoids found in the rocket accessions (Table 2). HGC1 accession (Figure 2) showed the maximum mean value for quercetin-3-β-glucoside (1680.0 µg g^{-1} dw). Rutin was found ranging from 12.00 to 27.00 µg g^{-1} dw in rocket accessions (Table 2).

Differences were found among the accessions of rocket in their mean flavonol contents. Thus, quercetin was only detected in HGC1 accession (13.50 µg g^{-1} dw).

This flavonol is a recognized supplement that could increase the nutraceutical value of rocket. Myricetin was found in the rocket leaves of the HGC1 and LGC2 accessions (3.00 µg g^{-1} dw) (Table 2).

Previous studies have shown discrepancies in phenolic content of rocket accessions. Thus, some works have shown that kaempferol di-O-glycoside was the major flavonoid in young *Eruca* leaves,[17] while other authors have reported kaempferol, isorhamnetin and quercetin as the predominant flavonoids.[21] Quercetin disinapoyl tri-O-glycoside has also been identified as predominant flavonoid in rocket accessions,[22] but it is worth mentioning that plants analysed were not *E. sativa* or they were an Italian ecotype with a very different leaf flavonoid profile to common commercially available *E. sativa*.[9] The variability observed in the phenolic profile between studies and plant varieties could be the result of multiple factors, including methodology (all reports used different approaches for extraction, chromatography, and quantification), sample characteristics and conditions, including variables such as growth.[23]

Among the phenolic acids, ferulic acid was identified varying from 30.60 (LGC2) to 48.00 µg g^{-1} dw (HGC1) in the rocket accessions (Table 2). This important phenol has been widely used as an immune-promoting agent in the treatment of blood deficiency diseases.

Our results showed variability for the mean content of total phenolic compounds (4474.5 to 32700 µg g^{-1} dw) between rocket accessions and demonstrated that rocket leaves, especially from LGC1 and HGC2 accessions, are an excellent source of these compounds. Both accessions reached higher values than those found in other sprouting species such as broccoli (27962.5 µg g^{-1} dw approximately)[24] and even richer than the commercial broccoli florets.[25] Nevertheless, LGC2 and HGC1

were the accessions that provided more qualitative variability regarding the phenols studied.

Carotenoid content in rocket leaves

Lutein was the principal carotenoid, followed by β-cryptoxanthin, β-carotene, zeaxanthin and violaxanthin (Table 3). Neoxanthin was found in lower proportions (less than 1% of total carotenoid content). The total carotenoid content ranged from a minimum mean value of 19.51 µg g^{-1} dw (LGC1 accession) to a maximum mean value of 263.91 µg g^{-1} dw (LGC2 accession). HGC1 and LGC1 accessions showed the maximum and minimum mean values for lutein (124.30 and 8.30 µg g^{-1} dw), respectively. LGC2 exhibited the highest β-carotene concentration with a mean content of 20.71 µg g^{-1} dw. All the rocket accessions had zeaxanthin and β-cryptoxanthin, and this is the first report on quantitative analysis of both carotenoids. β-Cryptoxanthin has only one-half of the provitamin A activity of β-carotene, but in many accessions of rocket (LGC1, LGC2 and HGC1), a higher concentration of the former than that of β-carotene has been found. Of the rocket accessions analyzed, LGC2 and HGC1 (Figure 3) had the highest concentrations of neoxanthin, violaxanthin, lutein, zeaxanthin and β-cryptoxanthin. The LGC1 accession presented the lowest levels of these main carotenoids, whereas HGC2 had intermediate concentrations.

In previous works lutein and β-carotene were reported as the most abundant carotenoids with mean contents of 50 µg g^{-1} for lutein and 30 µg g^{-1} for β-carotene.[5] The contents of lutein in this research are higher than those found in previous works, but lower than the values found for β-carotene. It has been suggested that 6 mg of lutein per day may reduce the risk of age related macular degeneration in a percentage

of 43%.[26] This concentration is equivalent to consuming ~7 kg tomatoes, 2 salad bowls of spinach, or, as revealed by this study, ~ 320 g of fresh leaves of rocket. Although lutein is not a provitamin A, it is a more effective antioxidant than many other carotenoids. It inhibits in vitro lipid oxidation in a more efficient manner than β-carotene, α-carotene or lycopene.

Sugars in rocket leaves

Glucose, the primary photosynthetic product, was the predominant sugar in leaves (Table 4). In fact, this sugar represents >70% of the total soluble carbohydrates in rocket leaves. This is not surprising as glucose represents the major transport sugar in rocket and contributes significantly to osmotic adjustment. Sucrose, fructose, galactose, arabinose, and mannose were found at low concentration (Table 4). The capability of supporting prolonged water deficiency is known for rocket.[27] The activity of these substances is related to their ability to raise the osmotic potential of the cell. Similar results were also reported in *Eruca* by Ashraf,[27] who found that salt tolerant populations had significantly higher soluble sugars in their leaves that salt sensitive populations at varying salt levels of the growth medium.

CONCLUSIONS

In summary, the high myrosinase activity and high levels of isothiocyanates, phenols and carotenoids in some of the rocket accessions (LGC2 accession) could be transferred through genetic engineering or conventional breeding programs to commercial lines such as "LGC1" to increase its potential benefits on human health. However, further research is essential to evaluate the biological activity of these four accessions, assessing the possible non-desirable effect before planning strategies for

designing functional food and improving consumer's health.

ACKNOWLEDGMENTS

This work was supported by the project P06-AGR-02230 (CICE, Junta de Andalucía) and FEDER funds. Authors gratefully acknowledge the Institute of Food Research, Norwich, U.K., for provision of technical materials and support. They are also grateful to Dr Richarch Mithen, (Department of Natural Products and Health, IFR), Dr. Angeles Alonso-Moraga and Dr. Mª Dolores Luque de Castro, (University of Córdoba, Spain), for insightful advice in all aspects of the work. We would also like to thank Faculté des Sciences Agronomiques of Gembloux (Belgium), Dipartimento di Scienze Botaniche of Palermo, (Italy) and Botanischer Garten der Universität of Karlsruhe (Germany), for the supply of vegetal material. Finally, we thank Nicholas Davies for his help in grammatical revision of the manuscript.

REFERENCES

1 Juge N, Mithen RF and Traka M, Molecular basis of chemoprevention by sulforaphane: a comprehensive review. *Cel Mol Life Sci,* **64**: 1105–1127 (2007).

2 Bennett RN, Mellon FA, Botting NP, Eagles J, Rosa EAS and Williamson G, Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of *Eruca sativa* L. (salad rocket). *Phytochemistry* **61:** 25–30 (2002).

3 Kim SJ, Jin S and Ishii G, Isolation and structural elucidation of 4-(beta-D-glucopyranosyldisulfanyl)butyl glucosinolate from leaves of rocket salad (*Eruca sativa* L.) and its antioxidative activity. *Biosci Biotechnol Biochem* **68**: 2444–2450 (2004).

4 Barillari J, Canistro D, Paolini M, Ferroni F, Pedulli GF, Iori R and Valgimigli L,

Direct antioxidant activity of purified glucoerucin, the dietary secondary metabolite contained in rocket (*Eruca sativa* Mill.) seeds and sprouts. *J Agric Food Chem* **53**: 2475–2482 (2005).

5 Niizu PY and Rodríguez-Amaya DB, New data on the carotenoid composition of raw salad vegetables. *J Food Comp Anal* **18:** 739–749 (2005).

6 Lamy E, Schröder J, Paulus S, Brenk P, Stahl T and Mersch-Sundermann V, Antigenotoxic properties of *Eruca sativa* (rocket plant), erucin and erysolin in human hepatoma (HepG2) cells towards benzo(a)pyrene and their mode of action. *Food Chem Toxicol* **46**: 2415–2421 (2008).

7 Yehuda H, Khatib S, Sussan I, Musa R, Vaya J and Tamir S, Potential skin antiinflammatory effects of 4-methylthiobutylisothiocyanate (MTBI) isolated from rocket (*Eruca sativa*) seeds. *Biofactors* **35**: 295–305 (2009).

8 Alqasoumi S, Carbon tetrachloride-induced hepatotoxicity: Protective effect of 'Rocket' *Eruca sativa* L. in rats. *Am J Chin Med* **38**: 75–88 (2010).

9 Bennett RN, Carvalho R, Mellon FA, Eagles J and Rosa EAS, Identification and quantification of glucosinolates in sprouts derived from seeds of wild *Eruca sativa* L. (salad rocket) and *Diplotaxis tenuifolia* L. (wild rocket) from diverse geographical locations. *J Agric Food Chem* **55:** 67–74 (2007).

10 Clifford MN and Brown JE, Flavonoids and Health. In Andersen OM and Markham KR, (Eds.). *Flavonoids: Chemistry, biochemistry and applications*, Taylor and Francis Group Inc, New York (2006).

11 Van den Berg H, Faulks R, Fernando-Granado H, Hirschberg J, Olmedilla B, Sandmann G, Southon S and Stahl W, The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J Sci Food Agric* **80:** 880–912 (2000).

12 ISO norm. Rapeseed determination of glucosinolates content – Part 1: method using high-performance liquid chromatography. ISO 9167-1, 1–9 (1992).

13 Wathelet JP, Wagstaffe P and Boenke A, The certification of the total glucosinolate and sulphur contents of three rapeseeds (colza), CRMs 190, 366 and 367. Commission of the European Communities Rpt. EUR13339 EN (1991).

14 Singleton VL and Rossi JA, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* **16:** 144–158 (1965).

15 Heimler D, Isolani L, Vignolini P, Tombelli S and Romani A, Polyphenol content and antioxidative activity in some species of freshly consumed salads. *J Agric Food Chem* **55:** 1724–1729 (2007).

16 Tadmor Y, Larkov O, Meir A, Minkoff M, Lastochkin E and Edelstein M, Reversed-phase high performance liquid chromatographic determination of vitamin E components in maize kernels. *Phytochem Anal* **11:** 370–374 (2000).

17 Bennett RN, Rosa EAS, Mellon FA and Kroon PA, Ontogenic profiling of glucosinolates, flavonoids and other secondary metabolites in *Eruca sativa* (salad rocket), *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket) and *Bunias orientalis* (Turkish rocket). *J Agric Food Chem* **54:** 4005–4015 (2006).

18 Kim SJ and Ishii G, Glucosinolate profiles in the seeds, leaves and roots of rocket salad (*Eruca sativa* Mill.) and anti-oxidative activities of intact plant powder and purified 4-methoxyglucobrassicin. *Soil Sci Plant Nutr* **52:** 394–400 (2006).

19 Melchini A, Costa C, Traka M, Miceli N, Mithen R, DePasquale R and Trovato A, Erucin, a new promising cancer chemopreventive agent from rocket salads, shows anti-proliferative activity on human lung carcinoma A549 cells. *Food Chem Toxicol* **47:** 1430–1436 (2009).

20 Blaževic I and Mastelic J, Free and bound volatiles of rocket (*Eruca sativa* Mill.).

Flav Fragrance J **23:** 278–285 (2008).

21 Selma MV, Martínez-Sánchez A, Allende A, Ros M, Hernández MT and Gil M, Impact of organic soil amendments on phytochemicals and microbial quality of rocket leaves (*Eruca sativa*). *J Agric Food Chem* **58:** 8331–8337 (2010).

22 Weckerle B, Michel K, Balazs B, Schreier P and Toth G, Three new quercetin 3,3',4'-tri-O-β-D-glucopyranosides from the leaves of *Eruca sativa*. *Phytochemistry* **57:** 547–555 (2001).

23 Brown AF, Yousef GG, Jeffery EH, Klein BP, Wallig MA, Kushad MM and Juvik JA, Glucosinolate profiles in broccoli: Variation in levels and implications in breeding for cancer chemoprotection. *J Amer Soc Hort Sci* **127:** 807–813 (2002).

24 Pérez-Balibrea S, Moreno DA and García-Viguera C, Genotypic effects on the phytochemical quality of seeds and sprouts from commercial broccoli cultivars. *Food Chem* **125:** 348–354 (2011).

25 Vallejo F, Tomás-Barberán FA and García-Viguera C, Potential bioactive compounds in health promotion from broccoli cultivars grown in Spain. *J Sci Food Agric* **82:** 1293–1297 (2002).

26 Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA and Willett WC, Dietary carotenoids, vitamins A, C and E and advanced macular degeneration. *J Am Med Assoc* **272:** 1413–1420 (1994).

27 Ashraf M, Organic substances responsible for salt tolerance in *Eruca sativa*. *Biol Plantarum* **36:** 255–259 (1994).

FIGURE LEGENDS

Figure 1. General structure skeleton of glucosinolates differing only in the side chain -R.

Figure 2. Selected reaction monitoring chromatograms obtained after LC-MS/MS analysis of phenolic compounds in extract from HGC1 rocket leaves. **A,** Rutin; **B,** Quercetin 3-β-D glucoside; **C,** Ferulic Acid; **D,** Quercetin; **E,** Myricetin.

Figure 3. HPLC chromatogram obtained after LC-UV analysis of carotenoid compounds in extract from HGC1 rocket leaves. **1**, Neoxantin; **2**, Violaxanthin; **3**, Lutein; **4**, Zeaxanthin; **5**, β-Cryptoxanthin; **6**, β-Carotene.

TABLE LEGENDS AND TABLES

Table 1. Glucosinolates and isothiocyanates (mean±standard deviation) isolated from rocket leaves.

Table 2. Phenolic compounds (mean±standard deviation) isolated from rocket leaves.

Table 3. Carotenoid content (mean±standard deviation) isolated from rocket leaves.

Table 4. Carbohydrates (mean±standard deviation) isolated from rocket leaves.

Supplementary Table 1. Retention time, molecular weight and optimization of the MS/MS step for qualitative and quantitative determination of phenolic compounds from leaves of rocket.

Supplementary Table 2. Parameters of the GC–MS/MS method. Segment, ion preparation mode, retention time, molecular weight, quantifier and qualifier ions, excitation storage level and excitation amplitude for identification/quantification of sugars.