Food Bioscience 54 (2023) 102887

Contents lists available at ScienceDirect

Food Bioscience

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

Phenolic profile of whole seeds and seed fractions of lentils and its impact on antioxidant activity

Antonio Manco [a,1], Carmela Gerardi [a,1], Giuseppe Romano [a], Leone D'Amico [a], Antonio Blanco [b], Francesco Milano [a], Gian Pietro Di Sansebastiano [c], Rind Balech [d], Barbara Laddomada [a,*]

[a] Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Lecce, Italy
[b] Department of Soil, Plant & Food Sciences, Plant Breeding Section, University of Bari, Via Amendola 165/A, 70126, Bari, Italy
[c] Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Lecce, Italy
[d] International Center for Agricultural Research in the Dry Areas (ICARDA), Terbol, Lebanon

ARTICLE INFO

Keywords:
Lens culinaris
Seed fractions
Seed coat pattern
Flavonoids
Tannins
Phenolic acids

ABSTRACT

Seed color and size are the major traits influencing consumer's acceptability and market class of lentils worldwide. In this paper we assessed the in vitro antioxidant capacity of whole seeds, hulls, and cotyledons of five lentil varieties in relation to their phenolic profile. The samples were evaluated for total polyphenol content and different phenolic classes, such as condensed tannin content, total monomeric anthocyanins, and phenolic acids. Individual phenolic compounds, including flavonols, flavanols, flavones, anthocyanins, and phenolic acids, were further quantitatively investigated by HPLC-DAD. Total antioxidant capacity was evaluated by ABTS and ORAC assays, and a direct measurement (ABTSdir) was used to evaluate the antioxidant capacity of the bioactive compounds present in the whole-meal flours without extraction. The five genotypes showed considerable variations in their phenolic content and profile as well as antioxidant activities. The results showed a preferential accumulation of phenolic compounds with antioxidant activity in the hulls compared to cotyledons. Delphinidin and cyanidin were the most abundant flavonoids in the hulls, while epicatechin and catechin were the most concentrated in the cotyledons. A highly significant correlation was observed between ABTS, ORAC and ABTSdir and total polyphenols. The antioxidant capacities were highly correlated with several individual phenolics detected in hulls and cotyledons. The overall results showed that the lentil fractions and extracts with higher phenolics had also higher antiradical activity which was independent on seed size and color. Identifying lentil genotypes with diverse phenolic profile in cotyledons and whole seeds could meet diverse consumers preferences and health requirements.

1. Introduction

Lentil (*Lens culinaris* Medik) is one of the oldest cultivated species worldwide being adapted to a variety of soils and environments including semi-arid marginal areas (Kaale et al., 2022). The global cultivation of lentils has increased in the recent years especially in Canada (2.87 Mt), India (1.18 Mt) and Australia (0.53 Mt) (FAO, 2021). Compared to other legume crops, lentil has higher annual growth with an average progression of about 10% over the last 60 years. Among the reasons of the lentil success is the high grain protein content (about 25%), that together with other grain macronutrients and micronutrients make this legume crop one of the most environmentally sustainable to meet food security (Kaale et al., 2022; Kiran et al., 2020). With respect to other pulses, lentils also contain a higher content of polyphenols especially of flavan-3-ols, condensed tannins (proanthocyanidins), anthocyanidins, flavonols, flavones, and phenolic acids (Dhull et al., 2022; Xu & Chang, 2007). These compounds, occurring in free, conjugated, and bound forms, are more concentrated in the peripheral layers of the seed (seed coat, or hull) than in cotyledons (Sun et al., 2020). A number of flavonoids and condensed tannins are the polyphenols that mostly contribute to grain color influencing also several organoleptic properties (Mirali et al., 2016, 2017).

The antioxidant activity of lentil polyphenols has been evaluated in vitro with different assays, such as 2,2′-azino-bis (3-

* Corresponding author. National Research Council, Institute of Sciences of Food Production (ISPA), via Prov. le Lecce-Monteroni, 73100 Lecce, Italy.
E-mail address: barbara.laddomada@ispa.cnr.it (B. Laddomada).
[1] The Authors contributed equally to this paper.

https://doi.org/10.1016/j.fbio.2023.102887
Received 19 May 2023; Received in revised form 13 June 2023; Accepted 25 June 2023
Available online 13 July 2023

ethylbenzothiazoline-6-sulfonic acid (ABTS) 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), ferrous reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and total radical-trapping antioxidant parameter (TRAP), among the most common (Xu & Chang, 2007). These methods are based on two main mechanisms, the hydrogen atom transfer (HAT) and the single electron transfer (SET). Indeed, due to the lack of standard methods for measuring the antioxidant capacity of food matrices, the use of different measurement methods is recommended (Zulueta et al., 2009). Polyphenols have other significant biological properties besides antioxidant activity, such as anti-inflammatory and anti-cancer properties associated to a reduced risk for cardiovascular diseases, diabetes, and neurodegenerative diseases (Calabriso et al., 2020; Ganesan & Xu, 2017; Jung et al., 2022; Peng et al., 2022).

Lentils are a staple in Middle Eastern and Indian diets but are at the base also of many traditional dishes around the world (Kaale et al., 2022). While lentils are mostly marketed as whole seed, a high percentage are consumed as dehulled footballs or split forms (Muehlbauer et al., 2009). Lentils with red cotyledon and small seeds are mainly consumed as dehulled in South Asia and in the Middle East and red dyes can be used to paint shelled lentils despite their toxicity to humans (Erdoğan, 2015; Muehlbauer et al., 2009). The removal of the outer seed coat improves the lentil digestibility and palatability resulting in a reduction of polyphenols and anti-nutritional factors (ANFs) such as lectins, trypsin enzyme inhibitors, phytates, saponins (Patterson et al., 2017; Romano et al., 2021). The seed coats derived from de-hulling are mostly used as animal feeding although they are rich in antioxidant bioactive phenolic compounds and have been used as food supplement (Dalgetty & Baik, 2006; Galgano et al., 2021; Portman et al., 2018).

Lentil seeds may have different colors with seed coats varying from light green to deep purple, tan, grey, brown, black or mottled, and cotyledon colors that can be yellow, orange, red, or green depending on the genotype and on the polyphenol composition of the seed coats and cotyledons (Ganesan & Xu, 2017; Mirali et al., 2017). The market acceptability of lentils is highly dependent on seed color. Lentils with uniform and green color are preferred in the United States and Europe, brown seeds are more appreciated in Italy and other Mediterranean countries, and shelled seeds with intense bright red color are the most consumed in India, Pakistan, Nepal, and Middle East countries (Erdoğan, 2015; Fiocchetti et al., 2009). A large variability for seed coat patterns is present in the lentil germplasm which has not been fully investigated on biochemical and microscopic basis (AGPG, 1985; Irakli et al., 2021).

In this paper we considered five varieties representative of the variability observed in a larger collection of pigmented lentils with yellow to orange cotyledons and seed coat background, and multiform seed coat pattern (Del Coco et al., 2022). The objectives were to characterize the seed fractions and whole seeds across the varieties to evaluate their in vitro antioxidant capacity in relation to the phenolic profile. The antioxidant capacity of the phenolic extracts was evaluated by ABTS and ORAC assays and by a direct ABTS assay (ABTS*dir*). The cuticle surface was studied by optical and confocal microscopy to inspect the preferential sites of pigments accumulation.

2. Materials and methods

2.1. Chemicals

Authentic reference standards of phenolic compounds (caffeic acid, *p*-coumaric acid, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid, vanillic acid, sinapic acid and ferulic acid, (+)-catechin, (−)-epicatechin, kaempferol, luteolin, myricetin, delphinidin and cyanidin), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, AAPH [2,2′-azobis (2-methyl-propionamide)] were obtained from Sigma-Aldrich (Milan, Italy), and Folin-Ciocalteu reagent, sodium hydroxide, formic acid and HPLC (high performance liquid chromatography) grade solvents were obtained from Carlo Erba Reagent (Milan, Italy).

2.2. Plant materials

Five lentil varieties, namely IG2580, IG5769, IG69497, IG129136, IG143547, with different pedigree, year of release, country of origin, and seed morphology were received by ICARDA, Lebanon (Supplementary Table 1 and Fig. 1). These genotypes were chosen for being representative of the variability for hulls and cotyledons color, hull pattern and seed size observed in a larger collection of lentil varieties (Romano et al., 2022). The plants were grown in the 2018/19 season at Terbol, Lebanon (33.81° N, 35.98° E), at ICARDA experimental station according to randomized complete block design including three replications (Shiv Kumar Shiv Kumar, ICARDA Lebanon, personal communication).

2.3. Dehulling and milling

Seeds (50 g) were soaked in distilled water 1:4 (seed:water,w/w) for 4 h at room temperature, then the water was drained out and stored while the seeds were manually dehulled. The hulls plus the soaking water, and the cotyledons were lyophilized. Dried seed fractions and whole seed samples were milled using a laboratory Mixer Mill MM400 (Reitsch, Düsseldorf, Germany) with a frequency of 30 Hz for 30 s.

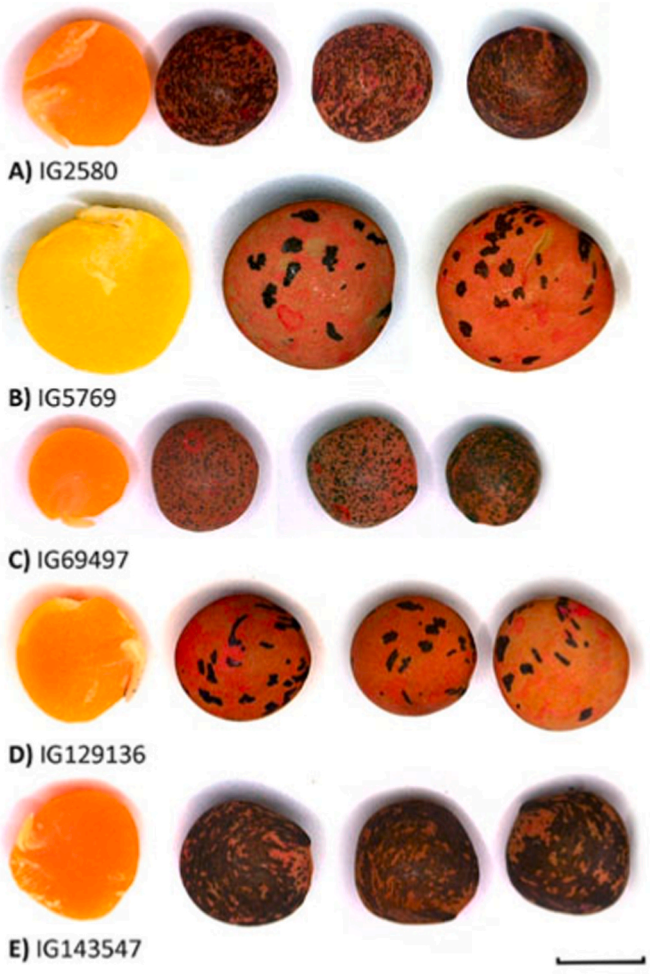

Fig. 1. Morphology and color of cotyledons (left) and hulls of five lentil varieties.

2.4. Morphological analyses

Lentils seed coat color was assessed using a Minolta CR-400 model spectrophotometer (Minolta Camera Co, Osaka, Japan) according to the International Commission on Illumination (CIELab, 1978). Whole seeds were transferred into the Minolta collector, and lightness (L*) and color (a*, red index; b*: yellow, index) were assessed. Seed coat patterns and cotyledons colors were classified based the descriptors reported by AGPG (1985).

Optical color pictures were acquired with the stereomicroscope Stemi 508 (Zeiss) connected to the axiocam ERc 5s camera. Microscopic optical analysis was performed with an optical microscope Orma Scientific OL201TL connected to a Camera Eurotek MDH5 (1080p). Confocal images were acquired with a laser scanning confocal microscope (LSM 710 Zeiss) using a λex:488 nm for excitation. Fluorescent emission was recorded combining 3 filters λem: 505–530; 560–615; >650 nm. Fluorescent emission was recorded combining 3 filters: 505–530; 560–615; >650 nm. Confocal sections have a 2.5 μm section at low magnification (Fig. 4A–F) and 1.5 μm section at high magnification (Fig. 4G–I). The power of each laser line, the gain, and the offset were identical for each experiment so that the images were comparable.

2.5. Extraction of total polyphenols and flavonoids analyses by HPLC

Total polyphenols were extracted following Gerardi et al. (2022) with slight modifications. Briefly, 0.1 g of whole-meal flours was extracted by adding 1 mL of methanol/water/formic acid (80/15/5) at room temperature and incubating overnight. Samples were centrifuged at 4000×*g* for 10 min, and the slurry was re-extracted with an additional 10 mL of solvent on a rotary shaker for 1 h, at room temperature. Sample extractions were performed in triplicate.

Flavonoids were extracted from hull, cotyledon and whole-meal samples using two different procedures according to Lee et al. (2017). Flavonoid-enriched extracts were obtained adding 1.2 mL of 50% methanol containing 1.2 M HCl to 0.1 g of seed fraction powder (hull and cotyledon). The samples were heated in a water bath at 80 °C for 2 h and then centrifuged at 10,000×*g* at 4 °C for 5 min. Anthocyanin-enriched extracts were achieved from 0.1 g of lentil hull or cotyledon seed powder by adding 0.95 mL of acidified methanol (85 methanol/15 HCl 1 N v/v) followed by sonication for 1 min. The samples were incubated at 38 °C for 5 min with a mixing frequency of 300 rpm, and subsequently hydrolyzed with 0.2 mL 6 N HCl at 90 °C for 2 h. The extracts were separated through centrifugation at 10,000×*g* at 4 °C for 5 min. All analyses were carried out in triplicate.

For flavonoids analysis the conditions were those described by Gerardi et al. (2020). The identification and quantification of individual phenolic acids was carried out following the conditions described in Alzuwaid et al. (2020).

2.6. Folin-Ciocalteu assay

Total phenol contents were determined following the method by Magalhães et al. (2010) and using a microplate reader (Tecan, Infinite M200). Folin-Ciocalteu reagent diluted in water from Milli-Q system (1:5 v/v) (50 μL) was placed in each well of a microplate, and then 100 μL of sodium hydroxide solution (0.35 M) was added. The absorbance value at 760 nm was recorder after 5 min of incubation. Gallic acid was used to obtain a calibration curve in the range from 2.5 to 40.0 mg/L. The total phenol content of each sample was expressed as gallic acid equivalents (GAE). The analyses were carried out in triplicate.

2.7. Determination of total anthocyanidins and condensed tannins

Total content of monomeric anthocyanidins (TACm) was determined on anthocyanin-enriched extracts using the pH differential method (Giusti & Wrolstad, 2001). The extracts were mixed using the appropriate dilution factor, with two different solutions at pH 1.0 pH 4.5 (Lee et al., 2005). TACm was expressed as cyanidin equivalents, was calculated according to the formula described in Hooshmand et al. (2021). The analyses were carried out in triplicate.

Condensed tannin content (CTC) was determined in the flavonoid-enriched extracts following the method used by Xu and Chang (2007). The amount of condensed tannin was calculated and expressed as mg catechin equivalents (mg of CAE/g sample) using the calibration curve of (+)-catechin. Linearity range of the calibration curve was 50–1000 μg/mL. The analyses were carried out in triplicate.

2.8. Phenolic acids extraction and analyses by HPLC

Total phenolic acids (the sum of the soluble and insoluble fractions) were extracted from 0.25 mg of tissues powders (hulls, cotyledons, and whole meal flours) through alkaline hydrolyses according to Hernandez-Espinosa et al. (2020). The ethyl acetate extracts were dried under nitrogen flux and dissolved in methanol:water (80:20 v/v). The analyses were carried out in triplicate.

A reversed phase-HPLC analytical method was used for the analysis of phenolic compounds. The apparatus was an Agilent-1100 liquid chromatograph (Agilent Technologies, Italy) equipped with a DAD detector (Agilent 1260 Infinity) and the separation was performed on a C18 column (5 μm UltraSphere 80 Å, 4.6 internal diameter x 250 mm length).

2.9. Total antioxidant capacity assays

ABTS assay was performed on total polyphenols extracts and flavonoid-enriched extracts according to Gerardi et al. (2021). The absorbance of samples was measured at 734 nm after 6 min using a plate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland). Antioxidant capacity values were expressed as Trolox equivalents (mmol Trolox Equivalent (TE)/g). Magellan v7.2 software (Tecan, Männedorf, Switzerland) was used to check the plate reader. The analyses were carried out in triplicate.

ORAC assay was carried out on total polyphenols extracts following the procedure established by Dávalos et al. (2004), using 96-well plates and an Infinite 200 Pro plate reader. The antioxidant Trolox was used to make a standard curve and values were expressed as mmol Trolox equivalents (TE)/g. The analyses were carried out in triplicate.

Total antioxidant capacity of whole-meal flours from the five lentil varieties were assessed following a direct procedure described in Romano et al. (2022) using the ABTS assay. The antioxidant capacity was expressed as mmol Trolox equivalent antioxidant capacity (ABTS-*dir*) per gram of whole-meal flour sample. The analyses were carried out in triplicate.

In this manuscript, the total antioxidant capacity obtained with the three different assays (ABTS on extracts, direct ABTS and ORAC) will be referred to as ABTS, ORAC and ABTS*dir*.

2.10. Statistical analysis

Analysis of variance for each trait was performed by using the software CoStat v. 6.4 (Ing, 1987). Pairwise Pearson correlation coefficients were calculated among all traits, except for cotyledon color for which Sperman rank correlation coefficients were determined. Results are given as mean ± standard deviation.

3. Results

3.1. Morphological traits of seeds and seed fractions

Lentil seed size and color are among the primary factors influencing consumer preferences (AGPG, 1985; Vandenberg & Slinkard, 1990). Different background color, pattern and cotyledon pigmentation

contribute to the overall grain color appearance (Xu & Chang, 2007). Based on seed diameter, lentil varieties have been classified into two main groups: microsperma (3–6 mm in diameter) and macrosperma (6–9 mm in diameter) (Barulina, 1930). Seed weight in lentil is generally expressed as hundred seed weight (HSW) and has been reported to influence also seed quality besides yield (Abbo et al., 1991).

In this study we considered five lentil varieties derived from breeding programs carried out in different countries which were released between 1991 and 2007 (Supplementary Table 1). Seed morphology varied among the varieties for seed coat color and pattern, cotyledon color and seed size (Fig. 1, Table 1). All the varieties were microsperma, except for IG5769 which was macrosperma (Table 1). HSW ranged from 2.14 g to 3.17 g among the microsperma varieties, while it was higher (5.54 g) in IG5769 (Table 1). Cotyledon color was brilliant orange for all the varieties except for IG5769 showing yellow cotyledon (Fig. 1). Based on lentil descriptors (AGPG, 1985), the seed coat patterns ranged from dotted (IG5769 and IG129136), spotted (IG69497), complex (IG2580) to marbled (IG143547) (Fig. 1).

Color parameters L*, a* and b* measured on the whole seeds of the five varieties are shown in Table 1. L values were significantly different ($p < 0.05$) among the varieties, with IG5769 having the highest value (50.74). The a* and b* values differed among the samples varying from 4.96 to 15.52 (a*) and 2.94 and 10.43 (b*), with IG5769 showing the highest values.

3.2. Pigments distribution in the seed coat

Seed coat (SC) optical imaging revealed that color was determined by different layers of cells. Similarly, to other microscopic description of *Lens culinaris*, the SC appeared to consist of multiple layers of cuticle cells, with a layer of sclereids underneath (Vohra et al., 2019). Darker thickening of the external cells surfaces characterized IG2580, IG129136 (Fig. 2A and E) and IG143547 (Fig. 3B). Additional colored metabolites were dispersed in the cell wall of deeper layers of SC. Non-dark areas of IG2580, IG5769 and IG69497 showed no dark thickening of superficial layers of SC (Fig. 2 A-B and C-D). In IG69497 SC, red or brown color could not be easily distinguished at macroscopic level probably because the color was determined by different overlapping of similar molecules. Darker areas in IG5769 (not shown) or IG129136 (Fig. 2E and F) showed again darker thickening of superficial layers of SC, but color could not be easily distinguished at macroscopic level from that of other varieties. Additional dark pigmentation was also due to dark aggregates inside the cell wall of deeper layers between cuticle and sclereids of SC (Fig. 2 G). Deeper in the SC, larger cells called sclereids were observed, deprived of evident pigmentation (Fig. 2G and H).

The superficial darker thickening contributed to dark color but was not fluorescent (Fig. 3B and C). The fluorescence usually associated with phenolics and, in particular, the emission above 600 nm generated exciting at 488 nm, was distributed in the cell wall of SC external cells. Fluorescence was emitted also from dark deeper areas so that correspondence of fluorescence derived from microscopic observations could not be correlated with macroscopically observed color. Dark areas of the SC in IG5769 were both optically dense and fluorescent (Fig. 3 E), with fluorescence emission at different wavelengths. Also, dark areas of the SC in IG143547 were fluorescent (Fig. 3 H). Interestingly, fluorescence and color were due to granular structures incrustating the cell wall (Fig. 3H and I). These observations indicated that phenolics compounds were mostly accumulated in the cell walls.

Table 1
Morphological traits of the seeds of 5 lentil varieties.

Variety	Color parameters of whole seeds			Cotyledon color	HSW[1] (g)	Size (mm)
	L*	a*	b*			
IG2580	45.68 ± 0.14^{d}	8.81 ± 0.11^{d}	6.1 ± 0.06^{c}	Orange	2.57 ± 0.02^{c}	4.1 ± 0.1^{c}
IG5769	50.74 ± 0.03^{a}	15.52 ± 0.03^{a}	10.43 ± 0.03^{a}	Yellow	5.54 ± 0.16^{a}	6.3 ± 0.1^{a}
IG69497	45.99 ± 0.08^{c}	9.85 ± 0.05^{c}	5.84 ± 0.01^{d}	Orange	2.14 ± 0.09^{d}	3.5 ± 0.1^{d}
IG129136	47.84 ± 0.04^{b}	10.46 ± 0.04^{b}	8.72 ± 0.04^{b}	Orange	3.17 ± 0.03^{b}	4.5 ± 0.1^{b}
IG143547	42.69 ± 0.02^{e}	4.96 ± 0.04^{e}	2.94 ± 0.04^{e}	Orange	3.09 ± 0.08^{b}	3.9 ± 0.1^{c}

Different letters within each column indicate significant differences ($p < 0.05$).
[1] HSW: hundred seed weight.

3.3. Phenolic contents and antioxidant activity of seed fractions and whole seeds

Lentils are particularly rich in flavan-3-ol, flavonols, anthocyanidins, condensed tannins and phenolic acids, which can vary for their content and composition among different varieties (Ahmmed et al., 2020; Sharma & Giri, 2022; Xu & Chang, 2010).

Total phenolic contents (TPC) of whole seeds from the five lentil varieties considered in this study are shown in Fig. 4. The values significantly varied among the varieties, with genotype IG5769 and IG2580 showing the highest (4.16 mg GAE/g and 4.13 mg GAE/g, respectively) and IG143547 the lowest values (2.20 mg GAE/g). Total antioxidant capacity of phenolic extracts from whole-meal flours varied across the varieties showing a similar trend to that of TPC (Fig. 4). Indeed, IG5769 and IG2580 had the highest ABTS*dir*, ORAC and ABTS values. Based on the different antioxidant assays, the resulting values showed a different magnitude with ABTS*dir* > ORAC > ABTS, except for IG129136 for which ABTS*dir* values did not vary significantly compared to the ORAC value suggesting that the bioactive molecules present in the whole-meal flour were the same reacting in the ORAC test.

The total polyphenol content of flavonoid-enriched extracts (TPF), total monomeric anthocyanins (TACm), condensed tannin content (CTC), and total phenolic acids (TPA) of seed coats (hulls), dehulled seeds (cotyledons) and whole seeds of the five lentil varieties are shown in Table 2.

TPF and TPA were 10-fold higher in the hulls than in cotyledons. The hulls also had 100-fold higher CTC than in the cotyledons and contained also TACm which being minor components could not be detected in cotyledons (Table 2). In general, the hulls of IG5769 contained higher TPF (57.17 mg GAE/g), CTC (397.56 mg CAE/g) and TPA (374.92 μg/g) compared to other varieties ($p < 0.05$). About cotyledons, again IG5769 was the variety showing the highest contents of most phenolic classes ($p < 0.05$). Table 2 shows the ABTS values of all phenolic extracts. The results indicated that the fractions with higher phenolics also had higher antioxidant activity.

3.4. Individual flavonoid and phenolic acids of seed coats and cotyledons

Flavonoids are generally classified into flavanols, flavanones, flavones, isoflavones, flavonols, and anthocyanidins and chalcones based on the oxidation state of the heterocyclic pyran ring C (Welch et al., 2008). Within each of these classes, individual compounds have different hydroxylation, and conjugation configurations (Beecher, 2003). In addition, many compounds may occur as aglycones or glycosidic forms which contribute to the complexity and number of flavonoids molecules (Harborne & Williams, 2000).

In this work, individual flavonoid aglycones were identified in the seeds and seed fractions of the five lentil varieties (Table 3). The results showed that delphinidin and cyanidin were the dominant compounds in the hulls. Second for abundance were the flavanol monomers catechin and epicatechin, followed by the flavonols kaempferol and myricetin, and the flavone luteolin. Among the cotyledons, the molecules that were most representative were epicatechin, followed by kaempferol and delphinidin. Myricetin was a minor compound which was detected only in the hulls. Significant variations ($p < 0.05$) for all the components were

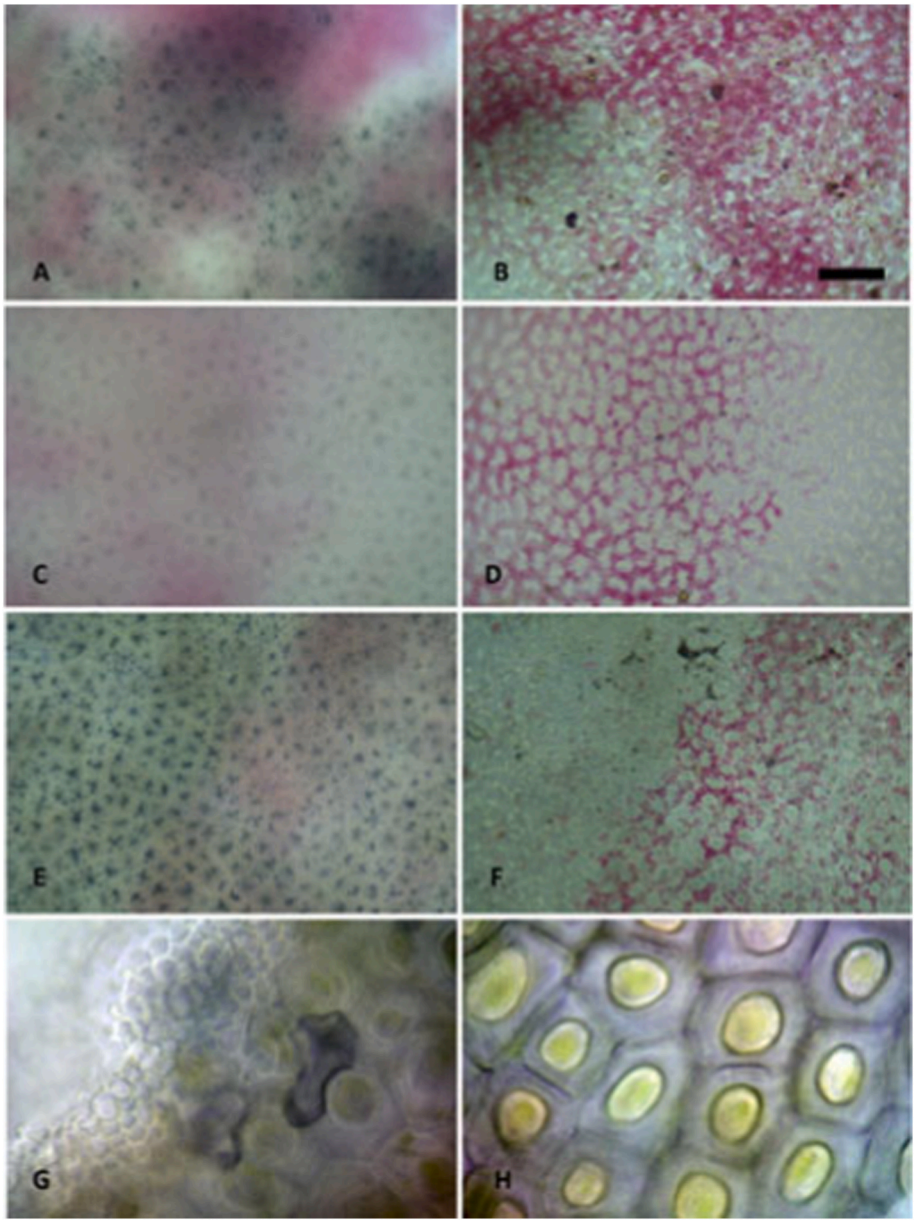

Fig. 2. Seed coat (SC) optical microscopy imaging. A) IG2580 SC surface; B) IG2580 SC surface with focusing on a deeper cell layer containing colored metabolites. C) non-dark areas of IG69497, with no dark thickening of the surface; D) IG69497 deeper cell layer containing colored metabolites. E) IG129136 darker thickening of superficial layers; F) IG129136 deeper cell layer containing colored metabolites. G) Additional dark pigmentation visible as dark aggregates inside the cell wall of deeper layers between cuticle and sclereids of SC. H) Deep sclereids of the SC.

observed among the genotypes both in the hulls and cotyledons. The hulls of variety IG5769 showed higher contents of delphinidin (2365 μg/g), catechin (1020 μg/g), epicatechin (456 μg/g), kaempferol (67.0 μg/g), and myricetin (10.1 μg/g) compared to other genotypes. The cotyledons were characterized by a different phenolic profile than the hulls, but IG5769 confirmed to be the variety with higher amounts of several compounds such as epicatechin (11,230 μg/g), kaempferol (215 μg/g) and delphinidin (38.0 μg/g) (Table 3).

The variation for individual phenolic acids among the hulls, cotyledons and whole seeds of the five lentils was also investigated (Supplementary Table 2). Phenolic acids are among the most abundant and ubiquitous metabolites of whole grain species (Liu, 2007). A total of nine phenolic acids were identified and quantified through HPLC-DAD in this study. The major phenolic acids found in the hulls were gallic acid (ranging from 217.45 μg/g to 346.96 μg/g) and protocatechuic acid (with contents from 114.02 μg/g to 144.14 μg/g), followed by *p*-coumaric acid > vanillic acid > syringic acid > ferulic acid > caffeic acid > sinapic acid. The phenolic acid profile of cotyledons showed some differences as compared to that of the hulls for both the composition and contents of compounds (Supplementary Table 2). Gallic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid were the most represented phenolic acids in cotyledons, followed by syringic, ferulic and sinapic acids.

3.5. Correlation between morphological traits and antioxidant activities

Correlation analyses between morphological traits and antioxidant activities are shown in Supplementary Table 3. As expected, highly significant positive correlations were found between seed size and seed weight. Positive correlations were detected also between seed size and the antioxidant activity of whole seed (*ws*) phenolic extracts with correlation coefficients ranging from $r = 0.54$ (ABTS*ws*), $r = 0.76$ (ORAC*ws*) and $r = 0.54$ (ABTS*dir*). All color parameters were strongly correlated between each other (data not shown).

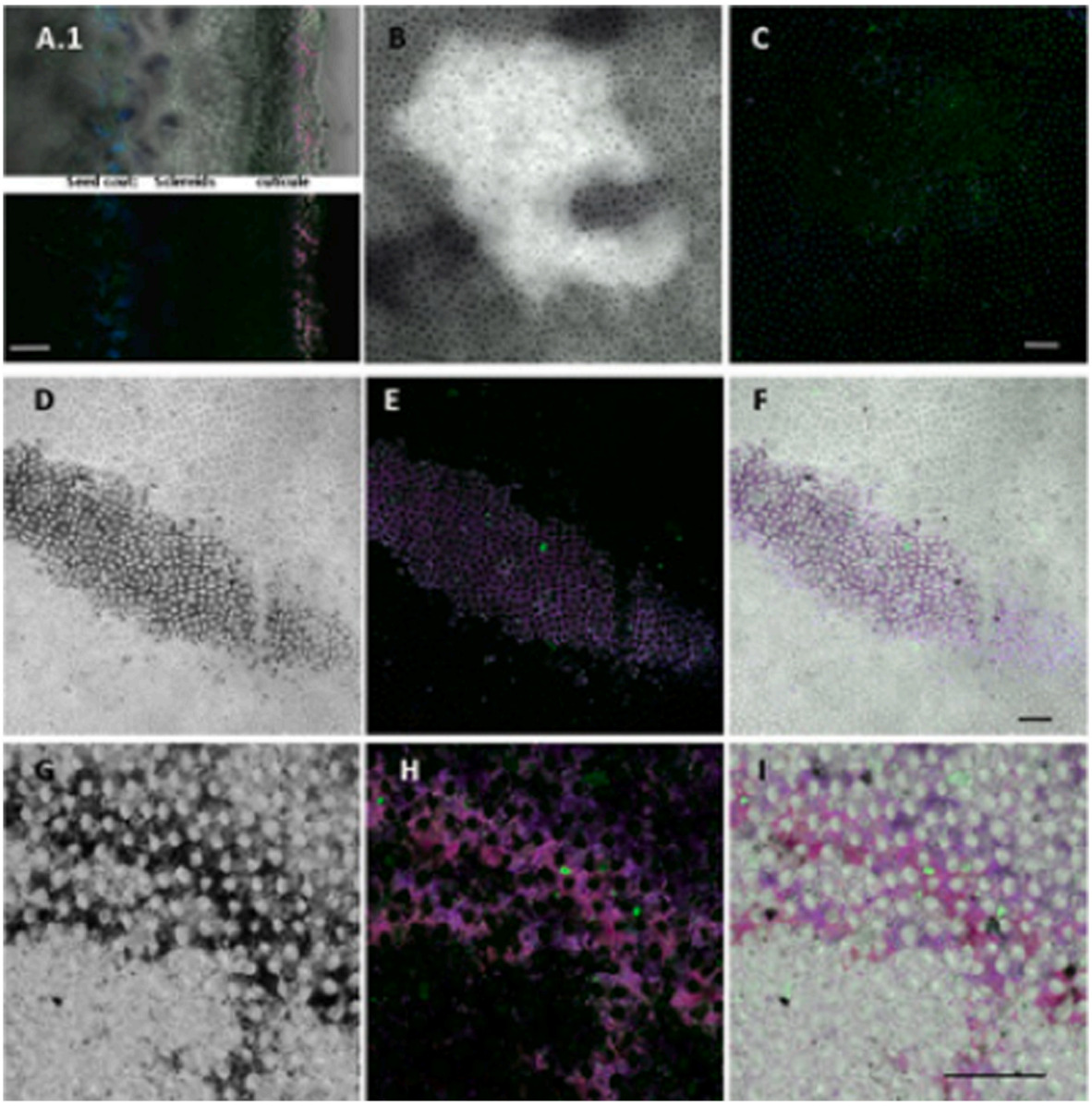

Fig. 3. Seed coat (SC) confocal imaging where it is evident that color do not correspond to fluorescence. A.1) Transmitted and fluorescent light imaging of the IG2580 line SC lateral section after separation from cotyledon, a multilayer seed coat can be visualized with wide spectrum fluorescent emissions (A.2) here detected at different wavelength: 505–530 (green), 560–615 nm (red), >650 nm (blue). B) Transmitted light image of dark areas of the IG143547 SC; C) fluorescence image of the same IG143547 SC frame, emission is limited to low wavelength (about 530 nm). D) Transmitted light image of dark areas of IG5769 SC; E) fluorescence image of the same IG5769 SC; F) combination of transmitted and fluorescent light imaging. G) Transmitted light image of dark areas of IG143547 SC; H) fluorescence image of the same IG143547 SC; I) combination of transmitted and fluorescent light imaging. Scale bar = 20 μm.

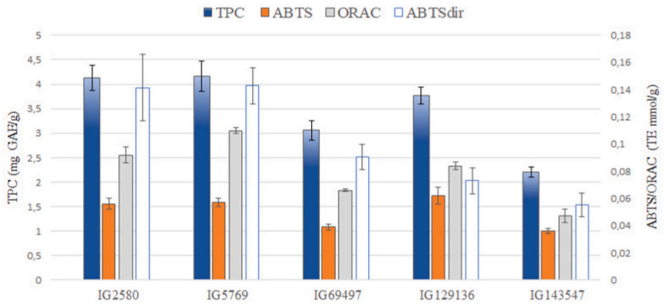

Fig. 4. Total phenolic compounds (TPC), and antioxidant capacity (TEAC, ORAC and TEAC dir, assessed by a direct procedure) of the whole-meal flours of 5 different lentil varieties. Error bars are standard deviations (SD) with n = 3.

3.6. Correlation between antioxidant activities, total polyphenols and individual phenolic compounds

Correlation analyses between antioxidant activities evaluated using different assays are shown in Table 4. Highly significant positive correlations were found between ORAC*ws* and ABTS*ws*, and between ORAC*ws* and ABTS*dir*. Moreover, the antioxidant activity of cotyledon (ABTS*c*) was correlated with ABTS*dir* ($r = 0.55$), ORAC*ws* ($r = 0.65$), and ABTS*h* ($r = -0.69$).

The correlations between total phenolic contents and antioxidant activities are summarized in Table 5. Among the phenolic extracts, TPC*ws*, TPF*h* and TACm*h* showed strong correlations with the antioxidant capacity. In detail, positive correlations were found between TPC*ws*, ABTS*ws*, ABTS*dir*, ORAC*ws* and ABTS*c*. In addition, TPF*h* was positively correlated with ABTS*dir* and ORAC*ws*, while negative

Table 2
Total phenolic compounds (TPF), total monomeric anthocyanins (TACm), total condensed tannins (CTC), total phenolic acids (TPA) and antioxidant capacity (ABTS) of the hulls, cotyledons, and whole seeds of 5 different lentil varieties.

Seed fraction	TPF (mg GAE/g)	TACm (mg CyaE/g)	CTC mg CAE/g	TPA (μg/g d.m.)	ABTS (TE mmol/g)
Hulls					
IG2580	52.50 ± 1.60^{b}	4.79 ± 0.38^{b}	302.36 ± 17.03^{c}	169.67 ± 19.37^{b}	0.59 ± 0.31^{c}
IG5769	57.17 ± 0.83^{a}	4.94 ± 0.12^{b}	397.56 ± 5.45^{a}	374.92 ± 2.81^{a}	0.79 ± 0.35^{a}
IG69497	48.27 ± 0.97^{b}	5.45 ± 0.0^{a}	344.96 ± 7.48^{b}	218.76 ± 52.38^{b}	0.73 ± 0.16^{b}
IG129136	48.38 ± 0.46^{b}	4.88 ± 0.1^{b}	343.63 ± 15.37^{b}	320.93 ± 9.57^{a}	0.69 ± 0.47^{b}
IG143547	50.92 ± 2.64^{b}	5.43 ± 0.13^{a}	346.44 ± 24.62^{b}	185.91 ± 5.78^{b}	0.81 ± 0.13^{a}
LSD	*3.47*	*0.35*	*28.40*	*65.55*	*0.06*
Cotyledons					
IG2580	5.02 ± 0.41^{a}	n.d.	3.96 ± 0.66^{b}	14.63 ± 0.11^{c}	0.063 ± 0.002^{a}
IG5769	5.04 ± 0.07^{a}	n.d.	4.99 ± 0.97^{a}	40.36 ± 3.81^{a}	0.058 ± 0.002^{ab}
IG69497	4.87 ± 0.41^{a}	n.d.	3.44 ± 0.28^{b}	16.66 ± 1.01^{c}	0.052 ± 0.001^{ab}
IG129136	5.05 ± 0.47^{a}	n.d.	4.91 ± 2.32^{b}	24.73 ± 3.98^{b}	0.061 ± 0.012^{ab}
IG143547	5.77 ± 0.03^{a}	n.d.	4.32 ± 0.67^{b}	11.79 ± 2.50^{c}	0.051 ± 0.001^{b}
LSD	*0.77*		*0.86*	*7.05*	*0.007*
Whole seeds					
IG2580	9.53 ± 0.44^{a}	0.47 ± 0.037^{b}	33.22 ± 2.18^{b}	29.84 ± 1.80^{c}	0.114 ± 0.001^{d}
IG5769	9.99 ± 0.14^{a}	0.47 ± 0.012^{b}	42.29 ± 0.55^{a}	78.14 ± 3.19^{a}	0.128 ± 0.002^{ab}
IG69497	8.02 ± 1.12^{a}	0.52 ± 0.002^{ab}	35.75 ± 0.62^{b}	35.78 ± 5.87^{c}	0.116 ± 0.002^{cd}
IG129136	10.46 ± 2.47^{a}	0.52 ± 0.012^{a}	41.15 ± 3.20^{a}	56.42 ± 2.53^{b}	0.126 ± 0.007^{ab}
IG143547	9.21 ± 1.24^{a}	0.50 ± 0.012^{ab}	35.74 ± 2.48^{b}	27.78 ± 2.80^{c}	0.121 ± 0.001^{cd}
LSD	*2.46*	*0.035*	*3.80*	*9.06*	*0.006*

Different letters show significant differences between the lentil varieties ($p < 0.05$). *LSD*: Least significant difference at $p < 0.05$.

correlation coefficients were observed between TACm*h* and antioxidant measurements.

The correlation between individual phenolic compounds detected in the hulls and cotyledons and antioxidant activities were also considered (Table 6, Supplementary Table 4). Gallic acid from hulls was significantly correlated with ORAC*ws*, while kaempferol correlated with ABTS*dir* and ORAC*ws*. Highly significant but negative correlations were found between cyanidin and antioxidant activities assay with ABTS*ws* and ORAC*ws*, and between luteolin and ABTSws and ORACws (Table 6). Among the phenolic compounds detected in cotyledons, again cyanidin was inversely correlated with all antioxidant capacity measurements (Supplementary Table 5). Similarly, *p*-hydroxybenzoic acid correlated with all antioxidant activity measurements but in a positive way.

Table 4
Correlation coefficients among total antioxidant activities evaluated by ABTS or ORAC assays on whole seeds (*ws*), hulls (*h*), and cotyledons (*c*) phenolic extracts. ABTS*dir*, antioxidant activity measured directly on whole-meal flour samples.

Variables	ABTS*ws*	ABTS*dir*	ORAC*ws*	ABTS*h*	ABTS*c*
ABTS*ws*	1				
ABTS*dir*	0.48	1			
ORAC*ws*	0.81***	0.79***	1		
ABTS*h*	−0.49	−0.40	−0.29	1	
ABTS*c*	0.79	0.55*	0.65**	−0.69**	1

*, **, *** Significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. n = 15.

Table 5
Correlation coefficients among whole seed (*ws*), hulls (*h*) and cotyledons (*c*) total phenolics and the antioxidant capacity evaluated by ABTS or ORAC assays. ABTS*dir*, antioxidant activity measured directly on whole-meal flour samples.

Variables	ABTS*ws*	ABTS*dir*	ORACws	ABTS*h*	ABTS*c*
TPC*ws*	0.87***	0.76**	0.94***	−0.50	0.73**
TPF*h*	0.27	0.64**	0.65**	0.24	0.25
TPF*c*	0.45	−0.16	0.06	−0.16	0.54*
CTC*h*	0.05	0.09	0.28	0.70**	−0.11
CTC*c*	0.22	−0.03	0.21	0.19	0.11
TACm*h*	−0.82***	−0.53*	−0.72**	0.47	−0.70**
TPA*h*	−0.47	−0.11	−0.19	0.56*	−0.54*
TPA*c*	−0.16	−0.38	−0.23	0.24	−0.30

TPC, Total phenolic content; TPF, Total polyphenols of flavonoid enriched extract; CTC, Condensed Tannin Content; TACm, Total monomeric Anthocyanins Content; TPA, Total Phenolic Acids.
*, **, *** Significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. n = 15.

Table 3
Flavonoids (expresses as μg/g dry matter) identified and quantified in the flavonoid extract of hulls, cotyledons and whole seeds of five different lentil varieties.

Variety	Delphinidin	Cyanidin	Catechin	Epicatechin	Kaempferol	Myricetin	Luteolin
Hulls							
IG2580	2343.27 ± 195.49^{a}	1864.68 ± 159.28^{b}	439.00 ± 21.14^{b}	230.25 ± 4.41^{d}	54.69 ± 4.12^{b}	6.56 ± 0.46 ^{c}	58.99 ± 3.20^{d}
IG5769	2542.62 ± 336.61^{a}	1780.02 ± 243.98^{b}	1020.52 ± 63.50^{a}	456.04 ± 23.24^{a}	67.00 ± 2.59^{a}	10.1 ± 0.18 ^{a}	68.44 ± 8.96^{c}
IG69497	2384.64 ± 35.77^{a}	2083.81 ± 52.79^{b}	266.70 ± 5.37^{c}	215.08 ± 6.23^{d}	43.44 ± 1.73^{c}	7.13 ± 1.30 ^{b}	85.74 ± 0.97^{b}
IG129136	2063.89 ± 71.09^{a}	1864.04 ± 66.04^{b}	414.26 ± 1.55^{b}	374.81 ± 16.84^{b}	36.79 ± 2.40^{d}	5.82 ± 0.42 ^{c}	44.51 ± 0.50^{a}
IG143547	2314.90 ± 45.78^{a}	2355.59 ± 30.30^{a}	342.73 ± 44.88^{bc}	298.96 ± 26.13^{c}	32.87 ± 3.89^{d}	9.38 ± 1.60 ^{a}	180.32 ± 2.13^{a}
LSD	*428.81*	*238.11*	*59.11*	*26.52*	*5.52*	*1.76*	*7.98*
Cotyledons							
IG2580	39.15 ± 0.55^{a}	7.86 ± 0.23^{b}	325.50 ± 14.54^{b}	7897.38 ± 180.94^{c}	140.01 ± 3.15^{c}	n.d.	2.66 ± 0.39^{b}
IG5769	37.97 ± 2.20^{a}	8.19 ± 1.38^{b}	284.83 ± 7.42^{c}	11232.45 ± 47.97^{a}	215.45 ± 17.17^{a}	n.d.	4.10 ± 0.47^{b}
IG69497	36.71 ± 0.99^{ab}	10.92 ± 0.86^{a}	242.72 ± 22.91^{d}	8817.29 ± 253.78^{b}	150.99 ± 16.43^{bc}	n.d.	6.58 ± 0.99^{a}
IG129136	33.49 ± 0.37^{b}	7.12 ± 0.92^{b}	450.20 ± 6.13^{a}	8524.01 ± 64.75^{b}	165.97 ± 4.62^{bc}	n.d.	4.21 ± 0.08^{b}
IG143547	36.34 ± 1.28^{ab}	12.37 ± 0.49^{a}	266.75 ± 8.61^{cd}	7606.83 ± 215.68^{c}	180.59 ± 16.80^{b}	n.d.	4.05 ± 0.04^{b}
LSD	*3.19*	*2.10*	*23.94*	*352.64*	*19.97*		*0.92*
Whole Seeds							
IG2580	265.12 ± 19.67^{a}	189.96 ± 15.83^{ab}	336.63 ± 12.97^{b}	7145.45 ± 162.98^{d}	131.64 ± 3.23^{c}	0.64 ± 0.05^{b}	8.18 ± 0.09^{d}
IG5769	275.93 ± 31.04^{a}	176.53 ± 22.34^{b}	354.73 ± 9.74^{b}	10209.75 ± 42.09^{a}	201.35 ± 15.63^{a}	0.95 ± 0.02^{a}	10.04 ± 1.27^{c}
IG69497	258.86 ± 4.12^{a}	207.04 ± 5.43^{ab}	244.99 ± 21.06^{d}	8003.21 ± 230.06^{b}	140.81 ± 10.57^{c}	0.67 ± 0.12^{b}	14.07 ± 0.95^{b}
IG129136	250.71 ± 7.28^{a}	205.78 ± 6.24^{ab}	446.35 ± 5.55^{a}	7652.18 ± 56.55^{c}	152.15 ± 4.05^{bc}	0.62 ± 0.04^{b}	8.52 ± 0.09^{d}
IG143547	245.62 ± 3.70^{a}	227.59 ± 2.49^{a}	273.72 ± 2.61^{c}	6935.62 ± 139.36^{d}	167.02 ± 10.60^{b}	0.86 ± 0.15^{a}	20.24 ± 0.16^{a}
LSD	*40.18*	*30.05*	*22.2*	*318.97*	*18.10*	*0.17*	*1.30*

Different letters show significant differences between the lentil varieties ($p < 0.05$).

Table 6
Correlation coefficients among individual phenolic compounds from the hulls (*h*) of five lentil varieties and total antioxidant activities (ABTS, ABTS*dir*, ORAC) of whole seeds (*ws*) phenolic extracts.

Variables	ABTS*ws*	ABTS*dir*	ORAC*ws*
DEL*h*	−0.59*	0.08	−0.20
CYA*h*	−0.79***	−0.81***	−0.97***
GAL*h*	0.71**	0.61*	0.85***
CAT*h*	0.48	0.63*	0.76***
EPI*h*	0.52*	0.15	0.54*
LUT*h*	−0.81***	−0.58*	−0.78***
KAE*h*	0.46	0.85***	0.84***
MYR*h*	−0.30	0.08	−0.02
SYR*h*	0.03	−0.07	−0.02
PRC*h*	−0.35	−0.59*	−0.47
VAN*h*	0.34	−0.33	0.06
CAF*h*	0.00	0.18	−0.04
COU*h*	0.65*	0.38	0.75**
FER*h*	0.06	0.10	0.25
SIN*h*	−0.89***	−0.44	−0.77**

DEL, delphinidin; CYA, cyanidin; GAL, gallic acid; CAT, catechin content; EPI, epicatechin content; LUT, luteolin; KAE, kaempferol; MYR, myricetin; SYR, syringic acid; PRC, protocatecuic acid; VAN, vanillic acid; CAF, caffeic acid; COU, *p*-coumaric acid; FER ferulic acid; SIN, sinapic acid.
*, **, *** Significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively; n = 15.

4. Discussion

Polyphenol content in lentils has been estimated to range from 1.02 to 24.34 mg GAE/g. Genotype and environmental factors can influence both the polyphenol content and composition (Ahmmed et al., 2020; Irakli et al., 2021; Sharma & Giri, 2022; Xu & Chang, 2010), but a large quote of variation depend also on different extraction methods (Galgano et al., 2021, 2023; Xu & Chang, 2007). Amarowicz et al. (2005) found differences for total polyphenol content when ethanol 95% or acetone: water solvents were used, the former extracting preferentially low molecular weight phenolics, the latter tannins and high molecular weight polyphenols. Acidified water-acetone, ethanol or methanol mixtures are among the best solvent to extract anthocyanins, flavonols, and hydroxycinnamic acid derivatives (Stanoeva et al., 2020; Xu & Chang, 2007). Thermal extraction can be used also to further enhance the yield of phenolics, however extraction time and temperature are critical to avoid decomposition and loss of bioactivity of flavonoids (Lee et al., 2017; Stanoeva et al., 2020).

In this study, we considered five lentil varieties with different seed size, seed coat background color and pattern, and cotyledon color which were representative of the variation observed in a larger lentil collection (Del Coco et al., 2022). The results showed that TPC ranged from 2.25 to 4.41 mg GAE/g (Fig. 2), while TPF (i.e. total polyphenols of flavonoid enriched extracts), were comprised between 8.02 and 10.46 mg GAE/g (Table 1). TPC were higher but comparable to the amounts found in fifteen lentil accessions analyzed by De Angelis et al. (2021). We also quantified the major classes of polyphenols in TPF extracts, such as CTC and total monomeric anthocyanins (TACm). Overall, all phenolic classes were more concentrated in the hulls, with CTC being 100-fold and TPF 10-fold higher than in cotyledons. Due to a dilution effect, whole seeds contained less polyphenols than the hulls, whereas compared to cotyledons they contained 2-fold more TPF and TPA, and 10-fold more CTC (Table 2), confirming previous literature findings (Dueñas et al., 2002; Mirali et al., 2014; Mishra et al., 2022).

Condensed tannins are among the major phenolics compounds in lentils (Amarowicz et al., 2005). Besides acting as antioxidants, they have been reported to exert adverse nutritional effects in humans being responsible of proteins precipitation, the inhibition of digestive enzymes, and complexing vitamins and minerals (Xu & Chang, 2007). CTC in lentils has been estimated to vary in a range of 0.12 and 16 mg CAE/g (Irakli et al., 2021; Menga et al., 2014; Xu & Chang, 2007). In this study we found higher values comprised between 33.22 and 42.49 mg CAE/g (Table 2) which could be explained by the different genotypes tested and environmental conditions (Irakli et al., 2021).

Analyzing the five lentil genotypes for total monomeric anthocyanins (TACm) we found a variation comprised between 0.47 and 0.52 mg/g in whole seeds which was higher compared to the results of previous works (Mishra et al., 2022; Xia et al., 2023).

Phenolic acids were less represented across the lentil varieties compared to flavonoids with contents ranging between 27.78 and 78.14 μg/g (Table 2). Overall, TPA resulted about 10-fold lower in cotyledons than in hulls. In fact, most individual phenolic acids were not detected in cotyledons or had lower contents than in hulls (Supplementary Table 2) in agreement with previous findings (Amarowicz et al., 2005; Dueñas et al., 2002; Mirali et al., 2014). Indeed, Xu and Chang (2010) found two times higher TPA compared to our results which could be explained by genotypic or environmental factors (Laddomada et al., 2021).

A total of seven individual flavonoid aglycone compounds were identified in the present study (Table 3, Supplementary Fig. 1). Compared to the molecules identified in the study of Lee et al. (2017), we detected and quantified also catechin and epicatechin both in the hulls and cotyledons. In this study, delphinidin and cyanidin were the most represented flavonoids in the hulls, while epicatechin and catechin were the most concentrated in the cotyledons (Table 3). Four individual flavonoids (i.e. delphinidin, cyanidin, myricetin and luteolin) were more abundant in whole seeds compared to cotyledons, while catechin, epicatechin and kaempferol had similar amounts between whole seeds and cotyledons (Table 2). These results suggested that the consumption of whole seeds results in a higher dietary intake of polyphenols.

All the antioxidant activity assays considered in the present study (ABTS, ORAC and ABTS*dir*) showed significant correlations with TPC, suggesting that they were reliable and effective to test the antioxidant activity of most polyphenols (Table 5). However, when flavonoid enriched extracts, only two out of four antioxidant capacity assays (i.e. ABTS*dir* and ORAC*ws*) significantly correlated with TPF*h* while no significant correlations were found between antioxidant measurements and TPF*c* (Table 5). That could be explained by the effect of the extraction procedure used for TPF that could had modified the bioactivity of phenolic compounds (Zulueta et al., 2009).

Many of the identified individual phenolic compounds contributed significantly to the antioxidant activities of phenolic extracts (Table 6 and Supplementary Table 4). This was supported by previous works showing that phenolics act as reducing agents, hydrogen donating antioxidants, mineral chelators, and singlet oxygen quenchers (Rudrapal et al., 2022; Scarano et al., 2023). In this study, the antioxidant activities of phenolic extracts were evaluated as radical scavenging capacity (ABTS), and oxygen radical absorbance capacity (ORAC). In addition, the antioxidant activity (ABTS*dir*) of the overall bioactive compounds bound to cell walls was tested without extraction but directly on the whole meal flours (Romano et al., 2022; Serpen et al., 2008). In fact, multiple methods of measurements are recommended to evaluate the antioxidant capacity of food's extracts or matrices (Zulueta et al., 2009). As a result, gallic acid and kaempferol quantified in the hulls were positively correlated with total antioxidant activities, while cyanidin and luteolin were negatively correlated (Table 6). The positive and negative correlation could be due to molecule concentration; in fact it is well known that based on their concentration, polyphenols can have antioxidant or prooxidant activity (Farhan & Rizvi, 2022).

The antioxidant activities assayed on TPC extracts differed largely among the varieties with IG280 and IG5769 having the highest values for all assays (Fig. 4). Overall, ABTS*dir* was higher compared to ORAC and ABTS, except for IG129136 (Fig. 4). This suggested that a different composition in phenolic compounds could result in different antioxidant mechanisms that are better evaluated using different assays. In previous studies, the ORAC value of lentil extracts was significantly influenced by the extraction solvents and genotypes (Khatun & Kim, 2021; Zou et al., 2011). ABTS and ORAC assays confirmed that lentil fractions and

extracts with higher phenolic and flavonoid contents have stronger antiradical action (Alshikh et al., 2015; Dueñas et al., 2006).

The specific histological localization of phenolic pigments was investigated partly supporting the biochemical analyses since autofluorescence provides a convenient approach with limited sample preparation (Albersheim et al., 2010; Fernández et al., 1999). Fluorescence may be predictive of characteristics depending on phenolics (Quintanilla-Casas et al., 2022). Unfortunately the λex:488 nm is not ideal for phenolics analysis but it excites mainly chlorophylls and phenol compounds related to lignins emitting from red to far red fluorescence and possibly hydroxycinnamic acids with a green fluorescence emission (Albersheim et al., 2010). We experienced that also several forms of flavonoids are excited by the λex:488 nm and emit red fluorescence (Faraco et al., 2017).

Flavonoids are mainly accumulated in epidermal tissues, in cell walls, vacuoles and nucleus but were also found in xylem vessel (Chen et al., 2017) and in cutin walls too (Bishnu & Moritz, 2017) so it was not surprising to find most of fluorescent compounds in cuticular layers of SC. Fluorescence emitted from cells of the cuticle may indicate the possible accumulation of flavonoids for UV-protection. The accumulation in the cell wall made their presence stable also during seed dehydration.

5. Conclusions

The seed coats, dehulled seeds, and whole grains of five pigmented lentil varieties were evaluated for the antioxidant capacity in relation to their phenolic profile. A preferential accumulation of phenolic compounds with antioxidant activity was observed in the hulls with respect to cotyledons. Indeed, whole grains contained 2-fold TPF and TPA, and 10-fold CTC than cotyledons. Among the individual phenolic compounds, delphinidin and cyanidin were the most abundant in the hulls, while epicatechin and catechin were the most concentrated in the cotyledons. ABTS, ORAC and ABTS*dir* were highly correlated with the phenolic composition of the hulls, especially with cyanidin, kaempferol, luteolin and gallic acid. In cotyledons, the antioxidant activities were highly correlated with cyanidin and *p*-hydroxybenzoic acid. Fluorescence with high λem, emitted from cells of the seed coat indicated the possible accumulation of flavonoids.

The results showed that independently on seed sizes and cotyledon color, seed coat background color and seed coat pattern, the genotypes IG2580 and IG5769 had the highest phenolic content and antioxidant activity both in the hulls and cotyledons. So far, identifying lentil genotypes with diverse phenolic profile in cotyledons and whole seeds is possible and could meet different consumers preferences and need for healthier lentils. In this framework, the use of hulls in human nutrition as food supplement could be enhanced considering their high phenolic contents, and antioxidant activity. Work is in progress in our laboratories to investigate on the genetic variability for phenolics in larger lentil collections across multiple environments and years to consider the huge variability for seed morphology in lentils.

CRediT authorship contribution statement

Antonio Manco: Writing – original draft, Formal analyses, Data curation. **Carmela Gerardi:** Writing – original draft, Formal analyses, Data curation. **Giuseppe Romano:** Formal analysis, Data curation. **Leone D'Amico:** Formal analyses, Data curation. **Antonio Blanco:** Writing – review & editing, Data curation. **Francesco Milano:** Formal analyses, Writing – review & editing, Data curation. **Gian Pietro Di Sansebastiano:** Formal analyses, Writing – original draft, Data curation. **Rind Balech:** Growing plant materials, Data curation. **Barbara Laddomada:** Conceptualization, Supervision, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The Authors declare that the work described in this article has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder.

Data availability

No data was used for the research described in the article.

Acknowledgments:

This research was partially funded by PRIMA Section 1 2020 Agrofood Value ChainIA Topic: 1.3.1-2020 (IA), MEDWHEALTH, project grant no. 2034. We would like to acknowledge Dr. Shiv Kumar and all ICARDA scientists, field workers, and lab assistants who helped in the collection of the samples used in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102887.

References

Abbo, S., Ladizinsky, G., & Weeden, N. F. (1991). Genetic analysis and linkage study of seed weight in lentil. *Euphytica, 58*, 259–266. https://doi.org/10.1007/BF00025258

AGPG. (1985). *AGPG. Lentil descriptor*. Rome, Italy: International Board for Plant Genetic Resources, 1985.

Ahmmed, T., Rahman, A., Salma, U., Akter, Z., Ansary, M. M. U., Khalil, M. I., Karim, N., & Bari, L. (2020). Nutritional, phytochemicals and antioxidant properties of some popular pulse varieties of Bangladesh. *Journal of Agricultural Chemistry and Environment, 9*, 343–368. https://doi.org/10.4236/jacen.2020.94025

Albersheim, P., Darvill, A., Roberts, K., Sederoff, R., & Staehelin, A. (2010). *Plant cell walls*. Garland Science.

Alshikh, N., de Camargo, A. C., & Shahidi, F. (2015). Phenolics of selected lentil cultivars: Antioxidant activities and inhibition of low-density lipoprotein and DNA damage. *Journal of Functional Foods, Natural Antioxidants, 18*, 1022–1038. https://doi.org/10.1016/j.jff.2015.05.018

Alzuwaid, N. T., Fellows, C. M., Laddomada, B., & Sissons, M. (2020). Impact of wheat bran particle size on the technological and phytochemical properties of durum wheat pasta. *Journal of Cereal Science, 95*, Article 103033. https://doi.org/10.1016/j.jcs.2020.103033

Amarowicz, R., Troszyńska, A., Baryłko-Pikielna, N., & Shahidi, F. (2005). Polyphenolics extracts from legume seeds: Correlations between total antioxidant activity, total phenolics content, tannins content and astringency. *Journal of Food Lipids, 11*, 278–286. https://doi.org/10.1111/j.1745-4522.2004.01143.x

Barulina, E. I. (1930). *Lentils of the U.S.S.R. and of other countries*.

Beecher, G. R. (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *Journal of Nutrition, 133*, 3248S–3254S. https://doi.org/10.1093/jn/133.10.3248S

Bishnu, P. K., & Moritz, K. (2017). Mechanical properties of cuticles and their primary determinants. *Journal of Experimental Botany, 68*, 5351–5367. https://doi.org/10.1093/jxb/erx265

Calabriso, N., Massaro, M., Scoditti, E., Pasqualone, A., Laddomada, B., & Carluccio, M. A. (2020). Phenolic extracts from whole wheat biofortified bread dampen overwhelming inflammatory response in human endothelial cells and monocytes: Major role of VCAM-1 and CXCL-10. *European Journal of Nutrition, 59*, 2603–2615. https://doi.org/10.1007/s00394-019-02109-y

Chen, C.-H., Xu, H., Liu, X.-H., Zou, L.-S., Wang, M., Liu, Z.-X., Fu, X.-S., Zhao, H., & Yan, Y. (2017). Site-specific accumulation and dynamic change of flavonoids in Apocyni Veneti Folium. *Microscopy Research and Technique, 80*, 1315–1322. https://doi.org/10.1002/jemt.22943

CIELab. (1978). International Commission on Illumination/Comission internationale de l'Eclairage/Internationale Beleuchtungskommission. https://cie.co.at/. (Accessed 16 May 2023).

Dalgetty, D. D., & Baik, B.-K. (2006). Fortification of bread with hulls and cotyledon fibers isolated from peas, lentils, and chickpeas. *Cereal Chemistry, 83*, 269–274. https://doi.org/10.1094/CC-83-0269

Dávalos, A., Gómez-Cordovés, C., & Bartolomé, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC−Fluorescein) assay. *Journal of Agricultural and Food Chemistry, 52*, 48–54. https://doi.org/10.1021/jf0305231

De Angelis, D., Pasqualone, A., Costantini, M., Ricciardi, L., Lotti, C., Pavan, S., & Summo, C. (2021). Data on the proximate composition, bioactive compounds, physicochemical and functional properties of a collection of faba beans (*Vicia faba* L.) and lentils (*Lens culinaris* Medik.). *Data in Brief, 34*, Article 106660. https://doi.org/10.1016/j.dib.2020.106660

Del Coco, M., Laddomada, B., Romano, G., Carcagnì, P., Kumar, S., & Leo, M. (2022). Characterization of a collection of colored lentil genetic resources using a novel computer vision approach. *Foods, 11*, 3964. https://doi.org/10.3390/foods11243964

Dhull, S., Kinabo, J., & Uebersax, M. (2022). Nutrient profile and effect of processing methods on the composition and functional properties of lentils (Lens culinaris Medik): A review. *Legume Science, 5*. https://doi.org/10.1002/leg3.156

Dueñas, M., Hernández, T., & Estrella, I. (2002). Phenolic composition of the cotyledon and the seed coat of lentils (Lens culinaris L.). *European Food Research and Technology, 215*, 478–483. https://doi.org/10.1007/s00217-002-0603-1

Dueñas, M., Hernández, T., & Estrella, I. (2006). Assessment of in vitro antioxidant capacity of the seed coat and the cotyledon of legumes in relation to their phenolic contents. *Food Chemistry, 98*, 95–103. https://doi.org/10.1016/j.foodchem.2005.05.052

Erdoğan, C. (2015). Genetic characterization and cotyledon color in lentil. *Chilean Journal of Agricultural Research, 75*, 383–389. https://doi.org/10.4067/S0718-58392015000500001

Faraco, M., Li, Y., Li, S., Spelt, C., Di Sansebastiano, G. P., Reale, L., Ferranti, F., Verweij, W., Koes, R., & Quattrocchio, F. M. (2017). A tonoplast P3B-ATPase mediates fusion of two types of vacuoles in petal cells. *Cell Reports, 20*, 2413–2422. https://doi.org/10.1016/j.celrep.2017.05.076. PMID: 28636930.

Farhan, M., & Rizvi, A. (2022). Understanding the prooxidant action of plant polyphenols in the cellular microenvironment of malignant cells: Role of copper and therapeutic implications. *Frontiers in Pharmacology, 13*, Article 929853. https://doi.org/10.3389/fphar.2022.929853

Fernández, S., Osorio, S., & Heredia, A. (1999). Monitoring and visualising plant cuticles by confocal laser scanning microscopy. *Plant Physiology and Biochemistry, 37*, 789–794.

Fiocchetti, F., Laddomada, B., Roselli, M., Crinò, P., & Lucretti, S. (2009). Fingerprinting of three typical macrosperma Italian lentil (Lens culinaris Medik.) landraces using fluorescence-based AFLP markers. *Scientia Horticulturae, 121*, 383–387. https://doi.org/10.1016/j.scienta.2009.02.006

Galgano, F., Condelli, N., Tolve, R., Scarpa, T., Caruso, M. C., Senizza, B., Marzario, S., & Lucini, L. (2023). Lentil seed coat as a source of phenolic compounds: Influence of geographical origin and genotype. *Food Measure, 17*, 1428–1437. https://doi.org/10.1007/s11694-022-01711-9

Galgano, F., Tolve, R., Scarpa, T., Caruso, M. C., Lucini, L., Senizza, B., & Condelli, N. (2021). Extraction kinetics of total polyphenols, flavonoids, and condensed tannins of lentil seed coat: Comparison of solvent and extraction methods. *Foods, 10*, 1810. https://doi.org/10.3390/foods10081810

Ganesan, K., & Xu, B. (2017). Polyphenol-rich lentils and their health promoting effects. *International Journal of Molecular Sciences, 18*, 2390. https://doi.org/10.3390/ijms18112390

Gerardi, C., D'amico, L., Migoni, D., Santino, A., Salomone, A., Carluccio, M. A., & Giovinazzo, G. (2020). Strategies for reuse of skins separated from grape pomace as ingredient of functional beverages. *Frontiers in Bioengineering and Biotechnology, 8*, 645. https://doi.org/10.3389/fbioe.2020.00645

Gerardi, C., Durante, M., Tufariello, M., Grieco, F., & Giovinazzo, G. (2022). Effects of time and temperature on stability of bioactive molecules, color and volatile compounds during storage of grape pomace flour. *Applied Sciences, 12*, 3956. https://doi.org/10.3390/app12083956

Gerardi, C., Pinto, L., Baruzzi, F., & Giovinazzo, G. (2021). Comparison of antibacterial and antioxidant properties of red (cv. Negramaro) and white (cv. Fiano) skin pomace extracts. *Molecules, 26*, 5918. https://doi.org/10.3390/molecules26195918

Giusti, M., & Wrolstad, R. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current protocols in food analytical chemistry*. https://doi.org/10.1002/0471142913.faf0102s00

Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55*, 481–504. https://doi.org/10.1016/s0031-9422(00)00235-1

Hernandez-Espinosa, N., Laddomada, B., Payne, T., Huerta-Espino, J., Govindan, V., Ammar, K., Ibba, M. I., Pasqualone, A., & Guzman, C. (2020). Nutritional quality characterization of a set of durum wheat landraces from Iran and Mexico. *LWT, 124*, Article 109198. https://doi.org/10.1016/j.lwt.2020.109198

Hooshmand, S., Mahdinezhad, M. R., Taraz Jamshidi, S., Soukhtanloo, M., Mirzavi, F., Iranshahi, M., Hasanpour, M., & Ghorbani, A. (2021). Morus nigra L. extract prolongs survival of rats with hepatocellular carcinoma. *Phytotherapy Research, 35*, 3365–3376. https://doi.org/10.1002/ptr.7056

Ing, S. J. (1987). *CoStat statistical software: CoHort software*. PO Box 1149, Berkeley, California 94701.

Irakli, M., Kargiotidou, A., Tigka, E., Beslemes, D., Fournomiti, M., Pankou, C., Stavroula, K., Tsivelika, N., & Vlachostergios, D. N. (2021). Genotypic and environmental effect on the concentration of phytochemical contents of lentil (Lens culinaris L.). *Agronomy, 11*, 1154. https://doi.org/10.3390/agronomy11061154

Jung, Y.-S., Lee, S.-H., Chun, S. Y., Kim, D. H., Jang, B. I., Han, M.-H., & Lee, S.-O. (2022). In vitro and in vivo protective effects of lentil (Lens culinaris) extract against oxidative stress-induced hepatotoxicity. *Molecules, 27*, 59. https://doi.org/10.3390/molecules27010059

Kaale, L. D., Siddiq, M., & Hooper, S. (2022). *Lentil (Lens culinaris Medik) as nutrient-rich and versatile food legume: A review* (p. e169). Legume Science n/a. https://doi.org/10.1002/leg3.169

Khatun, S., & Kim, T. (2021). Phenolic compound, antioxidant activity and nutritional components of five legume seed. *American Journal of Biomedical Science & Research, 12*, 328–334. https://doi.org/10.34297/AJBSR.2021.12.001767

Kiran, S., Johnson, J., Mani, J., Portman, A., Mizzi, T., & Naiker, M. (2020). Commercial lentils (Lens culinaris) provide antioxidative and broad-spectrum anti-cancerous effects. *Legume Research, 44*. https://doi.org/10.18805/LR-557

Laddomada, B., Blanco, A., Mita, G., D'Amico, L., Singh, R. P., Ammar, K., Crossa, J., & Guzmán, C. (2021). Drought and heat stress impacts on phenolic acids accumulation in durum wheat cultivars. *Foods, 10*, 2142. https://doi.org/10.3390/foods10092142

Lee, J., Durst, R. W., Wrolstad, R. E., & Collaborators. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal of AOAC International, 88*, 1269–1278. https://doi.org/10.1093/jaoac/88.5.1269

Lee, S.-Y., Yeo, Y.-S., Park, S.-Y., Lee, S.-G., Lee, S.-M., Cho, H., Chung, N.-J., & Oh, S.-W. (2017). Compositional analysis of lentil (Lens culinaris) cultivars related to colors and their antioxidative activity. *Plant Breeding and Biotechnology, 5*, 192–203. https://doi.org/10.9787/PBB.2017.5.3.192

Liu, R. H. (2007). Whole grain phytochemicals and health. *Journal of Cereal Science, The Contribution of Cereals to a Healthy Diet, 46*, 207–219. https://doi.org/10.1016/j.jcs.2007.06.010

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta, 83*, 441–447. https://doi.org/10.1016/j.talanta.2010.09.042

Menga, V., Codianni, P., & Fares, C. (2014). Agronomic management under organic farming may affect the bioactive compounds of lentil (Lens culinaris L.) and grass pea (Lathyrus communis L.)? *Sustainability, 6*, 1059–1075. https://doi.org/10.3390/su6021059

Mirali, M., Ambrose, S. J., Wood, S. A., Vandenberg, A., & Purves, R. W. (2014). Development of a fast extraction method and optimization of liquid chromatography-mass spectrometry for the analysis of phenolic compounds in lentil seed coats. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences, 969*, 149–161. https://doi.org/10.1016/j.jchromb.2014.08.007

Mirali, M., Purves, R. W., Stonehouse, R., Song, R., Bett, K., & Vandenberg, A. (2016). Genetics and biochemistry of zero-tannin lentils. *PLoS One, 11*, Article e0164624. https://doi.org/10.1371/journal.pone.0164624

Mirali, M., Purves, R. W., & Vandenberg, A. (2017). Profiling the phenolic compounds of the four major seed coat types and their relation to color genes in lentil. *Journal of Natural Products, 80*, 1310–1317. https://doi.org/10.1021/acs.jnatprod.6b00872

Mishra, G., Ankita, Aski, M., Tontang, M., Choudhary, P., Tripathi, K., Dhaka, A., Kumar, R., Thimmegowda, V., Stobdan, T., Atul, K., Bhardwaj, R., Praveen, S., Yadava, D., Kumar, S., & Dikshit, H. (2022). Morphological, molecular, and biochemical characterization of a unique lentil (Lens culinaris Medik.) genotype showing seed-coat color anomalies due to altered anthocyanin pathway. *Plants, 11*, 1815. https://doi.org/10.3390/plants11141815

Muehlbauer, F. J., Mihov, M., Vandenberg, A., Tullu, A., & Materne, M. (2009). Improvement in developed countries. *The lentil: Botany, Production and Uses, Botany, Production and Uses*, 137–154. https://doi.org/10.1079/9781845934873.0137

Patterson, C. A., Curran, J., & Der, T. (2017). Effect of processing on antinutrient compounds in pulses. *Cereal Chemistry, 94*, 2–10. https://doi.org/10.1094/CCHEM-05-16-0144-FI

Peng, L., Guo, F., Pei, M., Tsao, R., Wang, X., Jiang, L., Sun, Y., & Xiong, H. (2022). Anti-inflammatory effect of lentil hull (Lens culinaris) extract via MAPK/NF-κB signaling pathways and effects of digestive products on intestinal barrier and inflammation in Caco-2 and Raw264.7 co-culture. *Journal of Functional Foods, 92*, Article 105044. https://doi.org/10.1016/j.jff.2022.105044

Portman, D., Blanchard, C., Maharjan, P., McDonald, L. S., Mawson, J., Naiker, M., & Panozzo, J. F. (2018). Blending studies using wheat and lentil cotyledon flour—effects on rheology and bread quality. *Cereal Chemistry, 95*, 849–860. https://doi.org/10.1002/cche.10103

Quintanilla-Casas, B., Rinnan, Å., Romero, A., Guardiola, F., Tres, A., Vichi, S., & Bro, R. (2022). Using fluorescence excitation-emission matrices to predict bitterness and pungency of virgin olive oil: A feasibility study. *Food Chemistry, 395*, 133602. https://doi.org/10.1016/j.foodchem.2022.133602. Epub 2022 Jun 29. PMID: 35809549.

Romano, G., Del Coco, L., Milano, F., Durante, M., Palombieri, S., Sestili, F., Visioni, A., Jilal, A., Fanizzi, F. P., & Laddomada, B. (2022). Phytochemical profiling and untargeted metabolite fingerprinting of the MEDWHEALTH wheat, barley and lentil wholemeal flours. *Foods, 11*, 4070. https://doi.org/10.3390/foods11244070

Romano, A., Gallo, V., Ferranti, P., & Masi, P. (2021). Lentil flour: Nutritional and technological properties, in vitro digestibility and perspectives for use in the food industry. *Current Opinion in Food Science, 40*, 157–167. https://doi.org/10.1016/j.cofs.2021.04.003

Rudrapal, M., Khairnar, S. J., Khan, J., Dukhyil, A. B., Ansari, M. A., Alomary, M. N., Alshabrmi, F. M., Palai, S., Deb, P. K., & Devi, R. (2022). Dietary polyphenols and their role in oxidative stress-induced human diseases: Insights into protective effects, antioxidant potentials and mechanism(s) of action. *Frontiers in Pharmacology, 13*, Article 806470. https://doi.org/10.3389/fphar.2022.806470

Scarano, A., Laddomada, B., Blando, F., De Santis, S., Verna, G., Chieppa, M., & Santino, A. (2023). The chelating ability of plant polyphenols can affect iron homeostasis and gut microbiota. *Antioxidants, 12*, 630. https://doi.org/10.3390/antiox12030630

Serpen, A., Gökmen, V., Pellegrini, N., & Fogliano, V. (2008). Direct measurement of the total antioxidant capacity of cereal products. *Journal of Cereal Science, 48*, 816–820. https://doi.org/10.1016/j.jcs.2008.06.002

Sharma, K. R., & Giri, G. (2022). Quantification of phenolic and flavonoid content, antioxidant activity, and proximate composition of some legume seeds grown in

Nepal. *International Journal of Food Science, 2022*, Article 4629290. https://doi.org/10.1155/2022/4629290

Stanoeva, J. P., Balshikevska, E., Stefova, M., Tusevski, O., & Gadzovska Simic, S. (2020). Comparison of the effect of acids in solvent mixtures for extraction of phenolic compounds from aronia melanocarpa. *Natural Product Communications, 15*. https://doi.org/10.1177/1934578X20934675, 1934578X2093467.

Sun, Y., Deng, Z., Liu, R., Zhang, H., Zhu, H., Jiang, L., & Tsao, R. (2020). A comprehensive profiling of free, conjugated and bound phenolics and lipophilic antioxidants in red and green lentil processing by-products. *Food Chemistry, 325*, Article 126925. https://doi.org/10.1016/j.foodchem.2020.126925

Vandenberg, A., & Slinkard, A. E. (1990). Genetics of seed coat color and pattern in lentil. *Journal of Heredity, 81*(6), 484–488.

Vohra, K., Dureja, H., & Garg, V. (2019). Microscopic studies on Lens culinaris medikus seeds using bright-field microscope and polarized light microscope. *Plant Archives, 19*, 1980–1986.

Welch, C. R., Wu, Q., & Simon, J. E. (2008). Recent advances in anthocyanin analysis and characterization. *Current Analytical Chemistry, 4*, 75–101. https://doi.org/10.2174/157341108784587795

Xia, M., Li, M., Souza, T., Barrow, C., Dunshea, F., & Suleria, H. (2023). LC-ESI-QTOF-MS2 characterization of phenolic compounds in different lentil (Lens culinaris M.) samples and their antioxidant capacity. *Frontiers in Bioscience-Landmark, 28*, 44. https://doi.org/10.31083/j.fbl2803044

Xu, B. J. C., & Chang, S. K. C. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science, 72*, S159–S166. https://doi.org/10.1111/j.1750-3841.2006.00260.x

Xu, B., & Chang, S. K. C. (2010). Phenolic substance characterization and chemical and cell-based antioxidant activities of 11 lentils grown in the northern United States. *Journal of Agricultural and Food Chemistry, 58*, 1509–1517. https://doi.org/10.1021/jf903532y

Zou, Y., Chang, S. K. C., Gu, Y., & Qian, S. Y. (2011). Antioxidant activity and phenolic compositions of lentil (Lens culinaris var. Morton) extract and its fractions. *Journal of Agricultural and Food Chemistry, 59*, 2268–2276. https://doi.org/10.1021/jf104640k

Zulueta, A., Esteve, M. J., & Frígola, A. (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry, 114*, 310–316. https://doi.org/10.1016/j.foodchem.2008.09.033