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Phytochemical Analysis, *In Vitro* Antioxidant Activity and Germination Capability of Selected Grains and Seeds

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Abstract

The present research work was carried out to evaluate the antioxidant potential of several grains and seeds, such as chia, common oat, proso millet, amaranth, quinoa, buckwheat, flax seed, and einkorn. The antioxidant and radical scavenging activity was determined by using different *in vitro* assays. The phytochemical screening revealed that the studied grains contain flavonoids (from 27.80±0.72 to 159.39±2.17 μM QE/g DW), and phenolic materials (from 0.29±0.00 to 2.45±0.02 mg GAE/g DW), their content being in direct connection with the antioxidant activity. Sprouting capability was then characterized by use of conventional and hydroponic methods. The results obtained in the present investigation suggest that grains could be a potential daily source of bioactive substances.

Keywords: sprouting, phenolic profile, antioxidants, flavonoids, functional ingredients

1. Introduction

Food is the main energy source for the human body, and it needs nutritious food to be healthy. Grains are cultivated worldwide and comprise a big percentage of the world's daily meals. Food can be seen as a natural source of beneficial substances. Scientists have identified thousands of different phytochemicals (phenolic acids, flavonoids, etc.), which can aid in the treatment of different ailments. Various studies have shown that plants rich in phytochemicals may supplement the body's needs for free radical combat (Lobo *et al*., 2010) and attracting more and more attention (Georgieva-Krasteva *et al*., 2017; Popova and Mihaylova, 2017).

Grains should account for a sizable portion of the daily intake. There are different dietary recommendations for daily grain intake. One of them is serving 16 grams of whole grains. The grain consists of three main parts: bran, endosperm and germ. Most bioactive substances are in the bran (about 52 % by weight) and the germ (at least 24 % by weight) fractions. Non-refined grains have a lower glycemic index and are much more beneficial to the body providing it with sustained energy because they are released into the bloodstream more slowly (Zimmerman and Snow, 2012).

Phenolic compounds presented in cereals have gained much attention as health-promoting phytochemicals due to their strong antioxidant properties (Dapcevic-Hadnadev *et al*., 2018). They are secondary metabolites with different biosynthetic pathways, which are also associated with their ability to scavenge free radicals and chelate transition metal ions (Huyut *et al*., 2017). Some crops are a unique source of several compounds not present in other cereals and pseudocereals. Proso millet (*Panicum miliaceum* L.), mainly used as bird feed, has just recently gained attention for its substantial functional properties and beneficial constituents. This crop is reported to have superior nutritional properties such as high dietary fiber content, low glycemic index and rich micronutrient content (Chandel *et al*., 2014) and possess good antioxidant properties (Choi *et al*., 2007). Common oat (*Avena sativa* L.), a cereal of *Poaceae* family, is known for its rich polyphenol content, antioxidant and anti-inflammatory activities (Chu *et al*., 2013). Buckwheat (*Fagopyrum esculentum* Moench.), is primarily produced in Russia, and is known for its flavonoid content (Morishita *et al*., 2007). Einkorn (*Triticum monococcum* L.), nature's first and oldest wheat, can significantly contribute to an antioxidant intake with beneficial health effects (Lachman *et al*., 2012). Amaranth (*Amaranthus hypochondriacus* L.), highly valued for its amino acid composition also has considerable antioxidant activity (Tang and Tsao, 2017). Quinoa (*Chenopodium quinoa* Willd.) rich in phenolic acids, flavonoids, and tannins, is known to possess diverse physiological properties, including antimicrobial, antioxidant, anti-inflammatory, antitumor, and anti-carcinogenic effects (Benavente-Garcia and Castillo, 2008). Chia (*Salvia hispanica* L.) has high levels of phenolic compounds, flavonoids and antioxidant activity (Scapin *et al*., 2016). Flax seed (*Linum usitatissimum* L.) is rich in phenolic compounds, which are responsible for its antioxidant activities (Rubilar *et al*., 2010).

Germination is usually defined as the period when an organism grows from a seed. Germination process requires moisture, oxygen, temperature control and light/darkness. Sprouts are different vegetable/plant seeds or beans in a period of growth.

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Modern society is subjected to loads of stress, air pollution, climate change, modified products and artificial ingredients. It is reasonable for households to try to find ways to consume food that they trust. Germination is an easy and accessible way to add functional ingredients with beneficial effects to the daily diet. Healthy eating and back-to-nature food choices are trending. The reason why so many people use sprouts for food is that they contain a much higher percentage of vitamins and nutrients than the non-sprouted or mature forms of the abovementioned choices. They are low in calories, easily digestible and a valuable and important source of energy. Regular consumption of sprouts has an overall beneficial effect on health. Germinated seeds speed up metabolism and help reduce the amount of toxins in the body (Benincasa *et al*., 2019).

Previous work (Morishita *et al*., 2007; Chandel *et al*., 2014; Scapin *et al*., 2016) has only focused on characterizing the phytochemical profile of one or two species at a time as only few researchers have addressed the question of comparing several grains in terms of their beneficial properties. In the available literature, there was no information on the germination process of the studied seeds and grains. The objective of this paper was to investigate the phytochemical constituents profile and antioxidant activity of selective grains and seeds – einkorn, amaranth, common oat, buckwheat, proso millet, flax seed, quinoa and chia. First, the content of phenolic compounds and antioxidant capacities were evaluated in the extracts. Secondly, in order to characterize the samples antioxidant capacities, flavonoid content and phenolic acids have been determined. Finally, the sprouting capability of the studies grains and seeds was characterized with the use of two methods: conventional and hydroponic.

2. Materials and Methods

2.1. Materials and Extracts Preparation

Commercially available samples of eight species (chia, common oat, proso millet, amaranth, quinoa, buckwheat, flax seed, and einkorn) were obtained from local market in Plovdiv (Bulgaria) in spring 2018. The samples were milled into flour to obtain a homogenous particle size. Sample weighing 10g was extracted by stirring with 50 mL of methanol at 25°C at 150 rpm agitation for 24h and filtered through Whatman No. 4 paper. The procedure was repeated twice and the second extraction was carried out with 30 mL solvent. The extracts were pooled together and stored in refrigerator before analyzing.

2.2. Determination of Total Polyphenolic Content (TPC)

The TPC was analyzed following the method of Kujala *et al*. (2000) with some modifications. The TPC was expressed as mg gallic acid equivalents (GAE) per g dry weight of grains (DW).

2.3. Total Flavonoid Content (TFC)

The total flavonoid content was evaluated according to the method described by Kivrac *et al*. (2009).The absorbance was measured at 415 nm. Quercetin was used as a standard and the results were expressed as μM QE/g DW.

2.4. Determination of Antioxidant Activity

2.4.1. DPPH$^{\bullet}$ Radical Scavenging Assay

The ability of the extracts to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams *et al*. (1995) as described by Mihaylova *et al*. (2015).

2.4.2. ABTS•+ Radical Scavenging Assay

The radical scavenging activity of the extracts against 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was estimated according to Re *et al*. (1999). The results were expressed as TEAC value (μM TE/g DW).

2.4.3. Ferric-Reducing Antioxidant Power (FRAP) Assay:

The FRAP assay was carried out according to the procedure of Benzie and Strain (1999) with slight modification. Results were expressed as μM TE/g DW.

2.4.4. Cupric ion Reducing Antioxidant Capacity (CUPRAC) Assay

The CUPRAC assay was carried out according to the procedure of Apak *et al*. (2004). Trolox was used as a standard and the results were expressed as μM TE/g DW.

2.5. Identification and Quantification of Phenolic Acids

Qualitative and quantitative determination of phenolic acids was performed by using Elite LaChrome (Hitachi) HPLC system equipped with DAD and ELITE LaCHrome (Hitachi) software. Separation of the phenolic acids was performed by Supelco Discovery HS C18 column (5 μm, 25 cm×4.6 mm), operated at 30ºC under gradient conditions with mobile phase consisting of 2 % (v/v) acetic acid (solvent A) and acetonitrile (solvent B) as reported by Terzieva *et al*. (2017).

2.6. Germination

Seed samples were randomly selected from packed bags. Sixteen identical plastic containers were used as germination chambers. Eight chambers were filled with cotton wool soaked in deionized water and the remaining eight containers were filled with plant gel. Seed samples were spread in the containers. Germination was followed by daily counts, and final germination was determined after 5 days. A seed was considered germinated when the radicle protruded through the seed coat of at least 4 mm. Germination process was carried out at 25ºC and germination chambers were stored in the dark.

2.7. Statistical Analysis

Data were analyzed using MS Excel software. All assays were performed in at least three repetitions. Results were presented as mean ± SD (standard deviation). Fisher's least significant difference test at a level of $p < 0.05$ was used to determine the significance of differences between mean values.

3. Results and Discussion

The health benefits of whole grains consumption are often attributed to their unique phytochemical composition. Whole grain phytoconstituents are known for their antioxidant activity, and the ability to scavenge free radicals that may oxidize biologically relevant molecules (Liu, 2007). Based on this, grains could be considered as

contributors to the health benefits of people such as reducing the risk of heart disease, diabetes type 2, cancer, etc.

Phenolics are compounds consisting of one or more aromatic rings with one or more hydroxyl groups, generally categorized as phenolic acids, flavonoids, stilbenes, coumarins and tannins (Liu, 2004). Phenolics are the products of secondary metabolism in plants, providing essential functions in the reproduction and growth of the plant, acting as defense mechanisms against pathogens and parasites, also contributing to the color of plant. In addition to their role in plants, phenolic compounds in our diet provide health benefits associated with reduced risk of chronic diseases. Flavonoids on the other hand, include anthocyanins, flavonols, flavones, flavanones and flavonols. Flavonoids are located in the pericarp of all cereals. Cereals have only small quantities of flavonoids (McMurough and Baert, 1994). They are reported to have antioxidant, anticancer, anti-allergic, anti-inflammatory, anticarcinogenic and gastro protective properties (Cook and Sammans, 1996; Liu, 2004; Yao *et al*., 2004).

The results regarding the total phenol content and total flavonoid content of the methanol extracts of the selected grains are presented on Table 1. The TPC ranged from 0.29±0.00 to 2.45±0.02 mg GAE/g DW and the TFC was established to be from 27.80±0.72 to 159.39±2.17 μM QE/g DW. The total phenolic content was highest in chia (2.45±0.02 mg GAE/g DW), followed by common oat (1.82±0.02 mg GAE/g DW) and then buckwheat (0.76 ± 0.00 mg GAE/g DW). The lowest results according to both assays were evaluated for amaranth.

Table 1: Total phenolic content (mg GAE/g DW), total flavonoid content (μM QE/g DW) and *in vitro* antioxidant activity of grains methanol extracts (μM TE/g DW).

Sample /assay	TPC	TFC	DPPH	ABTS	FRAP	CUPRAC
chia	2.45 ± 0.02c	152.57±1.84a	5.63±0.04c	76.42±0.69cd	24.12±0.27a	15.83±21.80cd
common oat	1.82 ±0.02c	159.39±2.17a	1.23±0.01c	44.13±0.17cd	12.13±0.05b	20.61±23.54e
proso millet	0.68 ± 0.01c	36.67±5.05a	1.61±0.01c	<	1.56±0.03b	9.93±8.75e
einkorn	0.61±0.01a	29.16±2.21a	3.64±0.01c	64.02±0.75d	2.15±0.03e	10.38±2.67e
amaranth	0.29±0.00a	27.80±0.72a	0.57±0.01c	<	1.46±0.03e	6.76±7.84bcd
quinoa	0.69±0.64b	364.74±2.82a	3.34±0.06c	52.30±1.83d	6.32±0.08d	10.84±9.04bcd
buckwheat	0.76±0.00a	76.44±1.90a	3.64±0.04c	60.49±1.53d	6.14±0.11a	5.21±2.96e
flax seed	0.61±0.02c	83.60±4.24a	2.76±0.03c	60.19±1.61d	10.72±0.17 a	8.55±13.11cd

< below limit of detection; Means followed by different letters within a column are significantly different at $P < 0.05$ according to Fisher's LSD test.

In comparison, Akin-Idowu *et al*. (2017) established TPC in various amaranth species from 0.27 ± 0.011 to 0.31 ± 0.015 mg GAE/g grains corresponding to the currently reported results. These authors evaluated amaranth as a potent source of antioxidants since the reducing capacity of a compound is usually an indicator of its potential antioxidant activity. Marineli *et al*. (2014) reported total phenolic content of 0.94±0.06 mg GAE/g of chia seeds (Chile). The variations of the results in different research papers could be due to the concentration of phenolic compounds in whole-grain cereals influenced by grain types, varieties and the part of the grain sampled (Adom and Liu, 2002; Adom *et al*., 2003; Adom *et al*, 2005).

The investigation of antioxidant properties of natural sources is a very active field of research. Phenolic compounds have antioxidant properties and protect against degenerative diseases like heart diseases and cancer in which reactive oxygen species i.e., superoxide anion, hydroxyl radicals and peroxyl radicals are involved (Rhodes and Price, 1997). Based on this and the already established presence of phenolic compounds, the antioxidant activity of eight grains was assessed by four common assays in the current study (Table 1) because it is very difficult to select a single most suitable antioxidant assay method due to the diverse mechanisms of antioxidant action that no assay can capture in their entirety. The results varied significantly from 0.57±0.01 to 76.42±0.69 μM TE/g DW. According the DPPH assay, the highest results were evaluated for chia (5.63±0.04 μM TE/g DW), followed by buckwheat (3.64±0.04 μM TE/g DW) and einkorn (3.64±0.01 μM TE/g DW). In comparison, Inglett *et al*. (2010) reported 4.15±0.04 μM TE/g for buckwheat 100 % ethanol extract obtained by using a water bath at 50 °C.

ABTS scavenging activity ranged from 44.13±0.17 to 76.42 ± 0.69 μM TE/g DW. The highest values were established for chia (76.42±0.69 μM TE/g DW) and einkorn (64.02±0.75 μM TE/g DW). The lowest values were established for common oat - 44.13 ± 0.17 μM TE/g DW and proso millet and amaranth, where the antioxidant activity toward $ABTS^{\bullet+}$ was even not established. According to Akin-Idowu *et al*. (2017) the ABTS activity of five amaranth species varied from 169.6±3.77 to 201.5±4.04 mM TE/100g and Paśko *et al*. (2009) reported 19.63±1.38 mM TE/kg DW for amaranth grain. Despite this, amaranth is a valuable pseudo-cereal, due to its nutritional quality and nutraceutical properties, which contribute to improved human health (Gorinstein *et al*., 2007; Pasko *et al*., 2009).

The results with respect to the FRAP assay varied from 1.46±0.03 to 24.12 ±0.27 μM TE/g DW. The lowest results were evaluated in accordance with those already established in the amaranth extract (1.46±0.03 μM TE/g DW). The highest values were detected for chia and common oat - 24.12±0.27 and 12.13±0.05 μM TE/g DW, respectively. This corresponds to the values established in all conducted antioxidant assays.

According to CUPRAC assay, the antioxidant activity of the investigated grains ranged from 5.21±2.96 to 20.61±23.54 μM TE/g DW. The highest values were detected in common oat and chia samples - 20.61±23.54 and 15.83±21.80 μM TE/g DW, resp. The lowest

antioxidant potential was determined for buckwheat (5.21±2.96 μM TE/g DW) and amaranth methanol extracts (6.76±7.84 μM TE/g DW). Kumar and Kaur (2017) have evaluated the antioxidant capacity of 18 selected cereal crops. According to the CUPRAC assay, the values varied from 0.65 to 4.68 μM TE/g. Most studies concerning the antioxidant capacity of grains mainly target wheat, rice and rye products.

The most common phenolic compounds found in wholegrain cereals are phenolic acids and flavonoids. Phenolic acids are derivatives of benzoic and cinnamic acids and are present in all cereals. Hydroxybenzoic acid derivatives include p-hydroxybenzoic, protocatechins, vanillic, syringic and gallic acids. Hydroxyl cinnamic acid derivatives include p-coumaric, caffeic, ferulic and sinapic acids. The major phenolic acids in cereals are ferulic acids and p-coumaric acid (Hahn *et al*., 1983; Holtekjolen *et al*., 2006). Therefore, it seems reasonable to evaluate the phenolic acids profile of the investigated grains available on the Bulgarian market, which is the object of the present study (Table 2). The methanol extracts consisted of phenolic acids in wide range in total from 71.92±5.06 to 734.34±0.01 μg/g DW. The profile itself in some species was quite similar with respect to the investigated phenolic compounds. In particular, although the total phenolic acids content of einkorn and amaranth extracts differs the presented phenolic acids were the same. The highest content of the studied phenolic acids was established in quinoa extract - 734.34±0.01 μg/g DW, where the ferulic acid (495.59±0.06 μg/g DW) was predominant.

Table 2: Phenolic acids composition of grains methanol extracts (μg/g ~~g~~ DW).

sample/ compound	gallic acid	chlorogenic acid	caffeic acid	ferulic acid	p-coumaric acid	sinapic acid	total phenolic acids
Chia	14.27±0.01a	136.42±2.03a	34.63±0.01b	26.62±0.00c	-	168.58±0.01c	380.52±0.03a
common oat	13.88±0.00a	20.44±0.00a	15.10±0.00b	traces	21.91±0.01b	12.17±1.54b	83.5±0.01c
proso millet	6.88±0.01a	2.25±0.05a	5.37±0.01b	-	53.43±0.03a	8.29±0.01c	76.22±0.01a
Einkorn	19.19±0.07a	90.73±0.07c	6.95±0.03b	-	21.66±0.01ba	10.29±1.69b	148.82±1.23a
Amaranth	13.2±0.01a	22.79±0.08a	6.07±0.01b	-	21.64±2.07c	8.22±0.01c	71.92±5.06a
Quinoa	-	69.65±0.01a	19.42±0.06b	495.59±0.06c	108.27±0.0a1	41.41±0.00a	734.34±0.01b
Buckwheat	-	29.08±0.03c	19.41±0.01b	-	50.35±0.09b	-	98.84±0.04a
flax seed	13.54±0.03a	142.53±0.01c	5.42±0.05b	-	-	-	161.49±0.06c

"-" – not detected; "traces" – below limit of detection

Means followed by different letters within a column are significantly different at $P < 0.05$ according to Fisher's LSD test.

However, other research studies evaluated ferulic acid as the most abundant hydroxycinnamic acid found in cereal grains (Santiago *et al*., 2007; Boz, 2015). It is the main polyphenol present in cereals in which it is esterified to the arabinoxylans of the grain cell wall. Wheat bran is a good source of ferulic acid, which is esterified to the hemicelluloses of the cell walls (Dewanto *et al*., 2002). It has antioxidant properties to combat destructive free radicals, and astringency that deters consumption by insects and animals (Arnason *et al*., 1992). Ferulic acid can provide health benefits due to its antioxidant properties (Thompson, 1994).

However, in the present study chlorogenic and caffeic acids were distributed in all of the investigated extracts. On the contrary, the presence of cinnamic acid was not established in detectable amounts. In the chia extract, this compound was detected in traces, which corresponds to the results obtained by Coelho and Salas-Mellado (2014).

Germination is an easy and accessible way to add grains/seeds to the daily diet. For germination to occur, seeds/grains are soaked in water until seed splits open. Roots and shoot begin to occur after 24 h. Einkorn sprouts (Figure 1a) become ready in 72 hours. The conventional method led to a faster development compared to the hydroponic one. The hydroponic method resulted in several sprouts of less quality. Einkorn sprouts (cotton wool) had a length of 10-50 mm and hypocotyl thickness of 2 mm. They had yellow-rose color and attractive fragrance. They possess a slightly crisp texture, and for this reason can be purposely included in salads as functional ingredients. Benincasa *et al*. (2014) have also investigated the potential of einkorn sprouts as functional ingredients. Common oat sprouts (Figure 1b) did not develop very well. The conventional method resulted in a couple not fully developed sprouts and no sprouts in the hydroponic conditions. Amaranth sprouts (Figure 1c) developed for 72 hours in cotton wool. They had a length of 10-30 mm and hypocotyl thickness of 1 mm. They had a yellow-greenish color and possessed a leafy odor. They resembled the quinoa sprouts. The sprouting conditions did not alter the quality of the sprouts. Both conventional and hydroponic methods resulted in the same sprouts. Chia sprouts (Figure 1d) developed for 30 hours in cotton wool with a length of 40-50 mm, and did not develop in plant gel. They were white with green leaves and no particular odor. Flax seed sprouts (Figure 1e) developed for only 30 hours. They were light green of color with visible leaves. Their hypocotyl thickness was 2 mm. The conventional method in cotton wool led to faster development of the sprout. They possessed an attractive, broccoli like aroma and crisp texture. Quinoa sprouts (Figure 1f) did not develop very well in either condition. There were several sprouts in the cotton wool at a length of about 20-30 mm and a hypocotyl thickness of 0.8 mm. Their color was yellowish with an earthy odor and crispy texture. They are slightly like amaranth sprouts. Sprouts can find an application in the development of whole grain pasta with functional properties (Nataraja *et al*., 2018). Proso millet and buckwheat did not sprout in either condition. Since the samples were obtained from the local store, the inability of proso millet and buckwheat to germinate could be explained by many reasons i.e. storage conditions, integrity of the package, grains with sprouting inability, storage duration, etc.

(a) Einkorn sprouts conventional method (left), hydroponic (right)

(b) Common oat sprouts conventional method

(c) Amaranth sprouts conventional method (left), hydroponic (right)

(d) Chia sprouts conventional method

(e) Flax seed sprouts conventional method (left), hydroponic (right)

(f) Quinoa sprouts conventional method

Figure 1. Germination capability of selected grains and seeds.

4. Conclusion

Grains are accessible sources of phenolic compounds with potential health benefits. The studied extracts appeared to possess antioxidant activity. Among all investigated species chia, common oat and einkorn revealed the most potential with respect to total flavonoid content and antioxidant capacity. The polyphenol extraction technique applied revealed the most abundant phenolic acids to be chlorogenic and caffeic acids, while ferulic acid was detected in highest amounts. The highest total phenolic acid content was evaluated for quinoa (734.34±0.01 µg/g ǥ DW). The study demonstrates and corroborates the importance of whole grains as natural food antioxidants. The current findings add to the growing body of literature concerning whole grains and can improve the available knowledge in food data charts. The current results point out to the opportunity of further investigation the *in vitro* digestibility of the documented phytoconstituents as well as creating a nutrient profile of the sprouts.

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