

Comparative assessment of bioactive compounds and antioxidant activity of soft wheat bran on the Algeria market

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Abstract

From this context we have been interested in biochemical and phytochemical parameters of local and foreign varieties of wheat bran whose purpose is to search to see if there are intraspecific and interspecific varietal differences. The study concerned five varieties of soft wheat bran selected at CCLS Sidi Bel Abbés and consumed in many regions of Algeria. The biochemical compounds of grains (proteins, cellulose, ash content and phytochemical (total phenols, flavonoids and antioxidant activity) were determined in all varieties. The wheat bran, the subject and material of this study, was obtained from whole wheat grains collected from three varieties of soft wheat and used in our case to produce and extract the maximum of soft wheat bran. The varieties used were 'HD' (SWBHD) and 'Anzar' (SWBA) local variety and one imported, 'Habbour' variety (SWBHB). Two samples of marketed soft wheat bran, imported, were provided in this work to compare their nutritional components. Different techniques and methods were used in this experimental study (infra-red approach spectrophotometer, flame spectrophotometer). The results obtained show that 'Eriad' soft wheat bran (SWBE) is rich in protein with a variation of 15.78% at 18.07%; and is slightly elevated compared to other samples. Regarding the results obtained for cellulose, a high value was recorded for (SWBTAZ) variety 11.3%, and a lower one for (SWBHB) marketed at a value of 3.6%. The maximum concentration of potassium and sodium was obtained in the SWBAZ variety with a level of 3.16 mg/l and 30.36 mg/l respectively. The evaluation of phytochemicals has shown the presence of considerable amounts of polyphenols and flavonoids qualitatively and quantitatively. SWBAZ variety was very rich in total polyphenols, flavonoids and DPPH respectively with values of (1.101±0.01 mg EAG/g) and (0.174±0.001 EC/g) (1.39±0.01 EAG/g) compared to the different varieties studied. In conclusion, the SWBAZ variety could thus be considered, in our case, as an important source of phytonutrients.

Keywords: antioxidant activity; bioactive compounds; soft bran wheat; total phenols

Introduction

The importance of cereals at both global and national levels is undeniable as it is the basic of food for a large part of the world population but also for the feeding of farm animals. Algeria is also part of this logic of the fact that the basic culinary preparations should necessarily make use of cereals such as wheat, besides, in livestock feed rations. Wheat grains are rich in starch and proteins, which constitute an energy resource, and contain fibers, minerals and vitamins of group B, but also many phyto-micronutrients of phenolic compounds type (phenolic acids, alkylresorcinols, flavonoids, lignans and tannins), sterols combined or not with phenolic acids, carotenoids, betaine and choline (Curti *et al.*, 2013). Most of these compounds are found in greater abundance in the peripheral tissues of the grain (envelopes, layer aleurone and germ) and their quantities therefore decrease following processes of separation of the starchy albumen (heart of the grain) which precedes the manufacture cereal foods and aimed at obtaining products of sanitary and technological quality controlled, high and stable (Lullien-Pellerin, 2014). Wheat grain is an important cereal and staple in many parts of the world. Endosperm is the main nutritional component and is mined in crushing to produce basic ingredients such as flour and semolina.

The bran fraction is a by-product of milling (Curti *et al.*, 2013). Wheat bran, a by-product of wheat grain processing, consists of a stack of layers of cells (outer and inner pericarp, testa, epidermis and aleurone). At present, the value of wheat is poorly recognized despite its potential nutritional importance. It consists largely of the aleurone layer and therefore contains a high concentration of nutrients (Branlard, 2012). Hossain *et al.* (2013), indicates that wheat bran extract makes up about 13-19% of total wheat grain weight. Wheat bran is byproduct of the flour milling industry, but it is a great source of fibers, minerals, vitamin B6, thiamine, folate and vitamin E and antioxidants that are important for human health (Shewry, 2009). The extraction agent and method, the kind of cereals, the cultivar and the morphological structure and interactions between genotype and growing conditions are factors that influence the antioxidant activity that might significantly alter the properties of the wheat bran (Pinzino, 1999; Zielinski and Kozłowska, 2000; Marama *et al.*, 2004; Qu *et al.*, 2005; Kim *et al.*, 2006; Silva *et al.*, 2007). A study of Onyeneho and Hettiarachchy (1992) indicates that antioxidant activity is elevated by an increase in phenolic acids than other fractions of wheat, where we find the acid syringic, ferulic acid, vanillic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, composed major phenolic acid present in wheat bran. From this context we have been interested in biochemical and phytochemical parameters of local and foreign varieties of wheat bran and to see if there are intraspecific and interspecific varietal differences. The study concerned five wheat bran varieties selected and marketed from CCLS of Sidi Bel Abbés and consumed in many parts of Algeria.

Materials and Methods

Milling and bran extraction

The present study is devoted to the separation, analysis and comparison of local and imported soft wheat (*Triticum aestivum*) bran sold in the region of Sidi Bel Abbés northwest of Algeria. The number of studied samples is five (5) taken from the cereals and pulses cooperatives (CCLS), three varieties of soft wheat are used in our case to produce and extract the maximum of soft wheat bran, 'HD' (SWBHD) and 'Anzar' (SWBA) local variety and one imported variety 'Habbour' (SWBHB) (Table 1). Two samples of marketed soft wheat bran imported from the same region were provided in this part to compare their nutritional components with the provided unit ((SWBAZ) 'Azzouz' and 'Eriad' (SWBE) soft wheat bran). The wheat bran was obtained from whole wheat grains moistened for 24 hours. In this experimental part, we used the test mill at the 'Habbour' cv., which separates the bran from the other products. The three wheat cultivars were tempered to 16% moisture content and milled on a 'Habbour' mill following the method of Jeffers and Rubenthaler (1977). In this system, the grains are passed through counter-rotating corrugated metal rolls

producing break flour, middlings, and bran. Bran yields were measured at as-is moisture basis. Milling fractions were stored at -20 °C until the analysis.

Determination of chemicals parameters

Analysis in the infra-red approach (NIRS): Near-infrared spectrometry is an increasingly sophisticated analytical technique for the rapid control of grain quality. It allows to determine the rate of different parameters (moisture-ash-starch rate-rate of cellulose-protein level). It is a comparative analysis method based on the absorption of near-infrared light by organic matter (Alava *et al.*, 2001). The technique is based on the measurement of the reflectance of a radiation emitted at a given wavelength in the visible or the infrared light, the different chemical bonds of the tested product (OH, NH or CH) absorb it at lengths of specific wave, equal to their vibration frequency and thus go from a ground state to an excited state (Frédéric *et al.*, 2013).

Determination of minerals (determination of potassium and sodium)

For the screening of the mineral salts, 0.5 g of the sample powder was taken in 10 ml of distilled water, shaken for 1 hour and then centrifuged for 10 min. 2.43 g of KCl was taken in 100 ml of distilled water for the calibration curve of k+, but for the determination of sodium, 1.95 g of NaCl was taken in 100 ml of distilled water. Absorbance was measured by flame photometry.

Phytochemical study

Extraction of phenolics compounds

In our study the extraction of phenolic compounds was carried out according to Diouf *et al.* (2009), extraction method. Maceration was used as a reference extraction method. 10 g of each sample (wheat bran) was macerated in 100 ml of ethanol (70%). After 24 hours, the mixtures were separated by filtration. The extracts were then evaporated to dryness using the rotary evaporator at a temperature of about 45 °C.

Determination of total polyphenols

Folin-Ciocalteu reagent has the ability to oxidize the phenolate ions that result from the formation of the added sodium carbonate complex to the extract solution. A blue color is observed whose intensity reflects the concentration of phenolic compound in a given extract. The Folin-Ciocalteu method (Singleton *et al.*, 1999), simple and sensitive, is used to measure total phenols. In 100 µl of extract, 250 µl of diluted Folin reagent (50% v/v) (Sigma-Aldrich, Germany) was added. After 5 min of incubation at 25 °C, 250 µl of 20% (w/v) sodium carbonate was added into the tubes and the whole was brought to 2,000 µl with distilled water. The absorbance was read at 760 nm after 60 min. The whites were prepared for each variety by replacing the Folin reagent with distilled water. Gallic acid (Sigma-Aldrich, Germany) was used as standard and the results were expressed as mg gallic acid equivalent per 100 mg of dried material. Despite the sensitivity and simplicity of Folin method, it is not specific to polyphenols. Indeed, the reagent can react with proteins, reducing sugars, ascorbic acid and sulfur compounds (Singleton *et al.*, 1999).

Determination of flavonoids

The flavonoid assay is based on the formation of yellowish complexes by chelation of the Al³⁺ metals (used in the form of aluminum chloride (AlCl₃), by the hydroxyl groups of the flavonoids. The coloration thus formed is proportional to the flavonoid levels in the mixture (Ribereau-Gayon, 1968; Bahorun *et al.*, 1996). Flavonoid content was determined using a colorimetric method described previously by Jia *et al.* (1999). Briefly, 0.5 ml of the ethanol extract was diluted with 1 mL of distilled water, then, 0.075 ml of a 5% NaNO₂ solution was added to the mixture. After 6 min, 0.15 ml of a 10% AlCl₃ × 6 H₂O solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1M NaOH was added, and the volume was made up to 2.5 ml with distilled water. The solution was well mixed, and the absorbance was measured immediately against the blank (containing the extraction solvent instead of a sample) at 510 nm.

The flavonoids were quantified using a calibration curve obtained by measuring the absorbances of the known concentrations of the quercitrin spread solutions and the results were expressed in microgram equivalents of quercitrin per milligram of dry extract (mg EQ/g).

Determination of antioxidant activity

In order to evaluate the antioxidant activity of soft wheat bran skin extract, the following test was carried out: reducing power, radical scavenging DPPH reducing power (Oyaizu *et al.*, 1986).

DPPH radical scavenging activity

Diphenyl picrylhydrazyl (DPPH), a violet stable free radical in solution and having an absorbance at 517 nm, this colour rapidly disappears when the DPPH is reduced to diphenyl picryl-hydrazine by a compound with anti-radical property. The intensity of the staining is inversely proportional to the capacity of the antioxidants present in the medium to give protons (Sanchez-Morena, 2002). The DPPH scavenging activity was determined according to Abe *et al.* (1998) assay.

Antioxidant activity was measured by the DPPH method = 1,1-diphenyl-2-picrylhydrazyl (Sigma-Aldrich, Germany). To do this, 10 ml of a hydro-acetone solution (80%: 80 ml of acetone and 20 ml of water) was poured into tubes containing 0.5 g of flour (wheat bran) of each variety. The mixture was vortexed every 10 minutes for 2 h and centrifuged at 1700 g for 10 min. The supernatant was recovered for analysis. Ascorbic acid (Sigma-Aldrich, Germany) was used as a standard.

Antioxidant activity measured by the radical DPPH

This activity was determined by the method of Awika *et al.* (2003). The radical DPPH is dissolved in methanol at a concentration of $6.10 \cdot 10^{-5} \text{ mol.l}^{-1}$, kept at -20°C and protected from light before use. To each extract (0.3 ml) was added 2.7 ml of DPPH solution and the absorbance was measured at 517 nm. The results obtained were compared with ascorbic acid as standard. The IC₅₀ value represents the amount of powder providing 50% inhibition of DPPH.

Results and Discussion

Result of chemicals parameters

Isolation, identification and quantification of phytochemicals in wheat bran were conducted by (Liyana-Pathirana and Shahidi, 2005). This study deals with extraction methods which is different from one solvent to another in which acetone, ethanol and methanol are widely accepted solvents for extracting phenolic compounds. In our case, absolute ethanol has been used to prepare antioxidant extracts from wheat bran. The biochemical analyses carried out at the quality control laboratory level of the 'Habbour' mills by the INFRANEO infra-red spectrophotometer allowed us to study various parameters. The results obtained expressed in %, are shown in Table I. The moisture content is above the standards for all samples and represents from 13.09% to 17.07%. the ash content is consistent with the work of Curty (2013) and Oluwatoyin *et al.* (2015), and which varies from sample to sample with a value of 6.42% was observed for Soft wheat bran AZZOUZ (SWBAZ) and a rate of 3.43% registered for the Soft wheat bran 'Anzar' (SWBA). The results obtained show that 'Eriad' soft wheat bran (SWBE) is richer in protein with a variation of 15.78% at 18.07%; and it is slightly elevated compared to other samples; Protein values are consistent with the study of soft wheat bran (Curty, 2013; Yan *et al.*, 2015; Oluwatoyin *et al.*, 2015). The starch content is very high for Soft wheat bran 'HD' (SWBHD) 39.3%, 34.4% in Soft wheat bran 'Habbour' (SWBHB), 34.3% Soft wheat bran AZZOUZ (SWBAZ) samples, this may be due to the poor handling of the milling process, which causes the sound to contain fragments of the outermost layer of the starchy albumen, starch grains remain attached to the different particles of the sound (Reis *et al.*, 2006). On the other hand, a rate of

16% was recorded for soft wheat bran (SWBTAZ). Regarding the results obtained for cellulose, a high value was recorded for (SWBTAZ) variety 11.3%, is lower for (SWBHB) marketed at a value of 3.6%.

Table 1. Results in (%) of biochemical analyses of the different varieties of soft wheat bran

Variety	Origin	Humidity (%)	Ashes (%)	Proteins (%)	Starch (%)	Cellulose (%)
Soft wheat bran HD (SWBHD)	Algeria	15.45	3.8	17.66	39.3	4.5
Soft wheat bran Anzar (SWBA)	Algeria	17.07	3.43	16.66	34.3	4.8
Soft wheat bran Eriad (SWBE)	Import	13.31	3.95	18.07	34	5
Soft wheat bran Azzouz (SWBAZ)	French	13.09	6.42	17.36	16.4	11.3
Soft wheat bran Habbour (SWBHB)	Import	16.57	3.85	15.78	34.4	3.6

Results of determination of minerals (Na), (K) by flame spectrophotometer

The concentration of Na + and K + ions was calculated from the regression equation of the calibration ranges established with sodium and potassium (Appendix). We note from the results obtained in the Table 2; that varieties of soft wheat bran are richer in sodium than in potassium. The maximum concentration of potassium is in the (SWBA) variety with a concentration of 3.16 mg/l and a lowest concentration has been observed for Soft wheat bran (SWBHB) with an estimated value of 1.63 g/l. The high mineral content in the different varieties of samples of soft wheat bran is explained by the richness of the cereals in minerals, specifically the aleurone layer and the pericarp. A significant variability in the mineral content of one sample of wheat bran is noted in the literature, and these differences are influenced by environmental factors that characterize the cultivation of grain and wheat varietal effect of wheat grain (Pesterson *et al.*, 1986; Bock, 2000); the wheat grain transformation process is also a source of variation in the mineral concentration of the sounds (Bartnik and Jakubczyk, 1989).

Results of phytochemical analyses

Scientific research has focused on the extraction of phenolic compounds from new inexpensive or residual vegetable sources in the agro-food industries. The results of several studies confirm that phytochemicals of interest beneficial to consumer health are mainly found in bran and wheat germ (Vitagione *et al.*, 2008; Ivanisava *et al.*, 2012). The milling process commonly leads to product and isolating the starchy endosperm, the bran and germ portions are eliminating and destined for cattle feeding. The presence of the phenolics compounds in wheat bran, which are mainly covalently cross-linked with cell wall polymers (Adom *et al.*, 2005).

Table 2. Extraction yield in % ethanol / methanol of soft wheat bran samples

Variety	Methanol	Ethanol
Soft wheat bran HD (SWBHD)	10.68	19.46
Soft wheat bran Anzar (SWBA)	10.24	24.28
Soft wheat bran Eriad (SWBE)	14.24	30
Soft wheat bran Azzouz (SWBAZ)	11.21	14.14
Soft wheat bran Habbour (SWBH)	12.34	54.19

Efficiency of ethanol / methanol extraction

Extraction is a very important step in isolation, identification, use of phenolic compounds. The extraction methods depend on the extraction yield of the phenolic compounds (Ivanisava *et al.*, 2012). The extraction of phenolic compounds with ethanol in our samples allowed us to calculate the yield of each extract which was determined by 10 g of plant material expressed as a percentage. The results obtained are illustrated in the following Table 3. The results obtained by the ethanol extraction show that the (SWBH) extract has a strong yield at a value of 54.19%, other more or less considerable yield were observed in the extracts of (WBAZ) and (WBHB) respectively, a rate of 14.14% and 54.19% and finally the extracts of the (WBA) and (WBHD) local varieties represent respectively the lowest levels 24.28% and 19.46%.

Table 3. Concentration of potassium and sodium in mg in the five soft wheat bran samples

Variety	Potassium (K) in (mg)	Sodium (NA) in (mg)
Soft wheat bran HD (SWBHD)	2.44	29.84
Soft wheat bran Anzar (SWBA)	2.8	29.84
Soft wheat bran Eriad (SWBE)	1.63	26.15
Soft wheat bran Azzouz (SWBAZ)	3.16	30.36
Soft wheat bran Habbour (SWBH)	2.35	29.84

Determination of total polyphenols

Our results of the Folin-Ciocalteu reagent and the aluminum chloride colorimetric assay give us an idea of the content of phenolic compounds and total flavonoids. The phenolic extracts thus obtained generally have a honey-coloured, slightly caramelized pasty appearance for (SWBA) and (SWBAZ) extracts. The phenolic content was determined via the Folin-Ciocalteu test. The phenol content of each extract was then calculated from the calibration curve of gallic acid expressed in milligrams per gram of gallic acid equivalent dry matter. The experimental and predicted values for responses of compound phenolics different cultivars of bran wheat of extraction conditions are given in Figure I. The results showed that the total polyphenol of common wheat bran ranged from 0.081 to 0.13 mg (gallic acid equivalent - GAE)/g. The experimental values of PC were 0.921 mg GAE/g bran according to a descriptive study of Singh *et al.* (2012).

The results shown in Figure 1 show clearly that the amount of polyphenols is high in (WBAZ) 1.101±0.01 mg EAG/g, 0.911±0.4 mg EAG/g (WBHB) and 0.901±0.05 mg EAG/g (WBE) soft wheat extracts, followed by (WBA) and (WBHD) wheat bran extracts, which has the lowest level of polyphenols 0.843±0.82 mg EAG/g and 0.75±0.08 mg EAG/g. This variation can be explained in the differences that exist in the chemical composition between plant tissues. From a comparative point of view, the soft wheat bran of WBAZ and WBHB of French origin has the richest content in phenolic compounds than those of SBTA and SBTHD of local origin as well as WBHD. In addition, according to the work done by Oluwatoyin *et al.* (2015), the phenol compound was from 0.004 to 2.02 mg. Another study indicates that, the total phenolic contents in durum wheat bran were ranged from 7746.54 to 12384.55 mg GAE/kg. According to the study reported by Zilic *et al.* (2016), the content of acetone/water extractable phenolic compounds were ranging from 2700 to 3500 mg GAE/kg in the bran of 20 wheat genotypes. Genetic variation between varieties of the same species must be taken into account in order to evaluate phenolic compounds, because these compounds are dependent on the structure and genetic composition of the wheat raw material and also the method of extraction as well as production of wheat bran which causes a great variation between the cultivars. The layers of wheat bran contain several tissues (aleurone layer, intermediate layers, and seed coat) which contain essential nutrients, namely total phenolics which are mainly related to cell wall components (Adom and Liu, 2002; Beta *et al.*, 2005; Liyana-Pathirana *et al.*, 2006). Another work has shown that it is the aleurone layer (wheat bran fraction) in relation to other tissues which consistently has the highest antioxidant capacity among wheat fractions (Mateo Anson *et al.*, 2008; Vaher *et al.*, 2010).

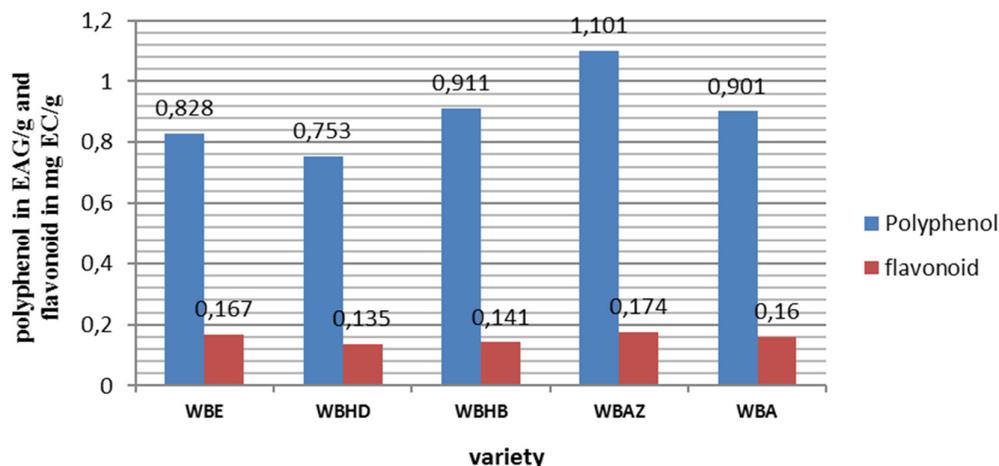


Figure 1. Concentration of polyphenols (in mg EAG/g) and flavonoids in mg EC/g of soft wheat bran varieties

The different varieties of soft wheat bran samples: SWBHD: Soft wheat Bran HD; SWBA: Soft wheat Bran Anzar; SWBE: Soft wheat Bran Eriad; SWBAZ: Soft wheat Bran Azzouz; SWBHB: Soft wheat Bran Habbour

Flavonoid dosage

Important polyphenolic class, with more than 5000 compounds already described (Gomez-Caravaca *et al.*, 2006). The flavonoid assay was performed according to the $AlCl_3$ method using quercetin as standard ($Y = 4.6153x - 0.0118$). The results obtained are expressed in milligrams per gram of dry matter in equivalent of quercetin. The flavonoid levels are presented in the Figure 2. It can be seen from the results that the amount of flavonoids in the WBAZ soft wheat bran extract is greater, followed by extracts of the WBA soft wheat bran, and WBE and the WBHB and WBHD extracts have the lowest high. The work of Oluwatoyin *et al.* (2015) and Brewer *et al.* (2014) estimates a flavonoid level of 3000-4300 micrograms. Flavonoids is a class of beneficial antioxidant substances of total phenolic compounds; some studies suggest that this class is more effective as antioxidants than vitamin C, according to results of Žilić *et al.* (2016). The flavonoids were detected, bound to the cell wall of wheat. Total flavonoids recorded in durum wheat bran was 259.31 mg CE / kg.

Scavenger power of the radical DPPH

The DPPH radical is a stable organic free radical, with a maximum absorption band between 515-528 nm. In this test the antioxidants reduce and discolour the radical DPPH, has a yellow compound diphenyl picryl hydrazine, the extent of the reaction will depend on the ability of antioxidants to give the hydrogen (Ardestani and Yazdanparast, 2007). The results can be expressed as a percentage of anti-radical activity or as a percentage of remaining DPPH (Bastos *et al.*, 2007). In this test, the highest antiradical power is that of vitamin C with a percentage of $(76.522 \pm 102.45\%)$. The SWBE sample had the highest anti-radical activity $(63.007 \pm 2.01\%)$, followed by SWBAZ $(40.325 \pm 2.45\%)$, and then the SWBHD sample with a rate of $(53.25 \pm 3.92\%)$. Values vary in the following order: SWBE < SWBAZ < SWBA < SWBHB < SWBHD-Ascorbic Ac. According to (Turkmen *et al.*, 2007) polyphenols appear to be efficient donors of hydrogen to the DPPH radical, due to their ideal structural chemistry.

The IC₅₀ of a compound is inversely related to its antioxidant capacity because it expresses the amount of antioxidant required to reduce the DPPH radical to 50%. A low IC₅₀ indicates the highest antioxidant activity (Zhang *et al.*, 2010; Senthilkumar *et al.*, 2012; Benadjabeur *et al.*, 2012). Our results reveal that all extracts have a good antioxidant activity whose IC₅₀s vary between 53.25 ± 26.62 and 163.007 ± 0.77 $\mu\text{g/ml}$ (Figure 3). The antioxidant activity of our samples could be attributed to their richness in phenolic compounds; the flavonoids have an ideal structure for the free radical scavenging, since they have

a number of hydroxyls acting as hydrogen donors, which in turn makes important antioxidants (Usmani *et al.*, 2013).

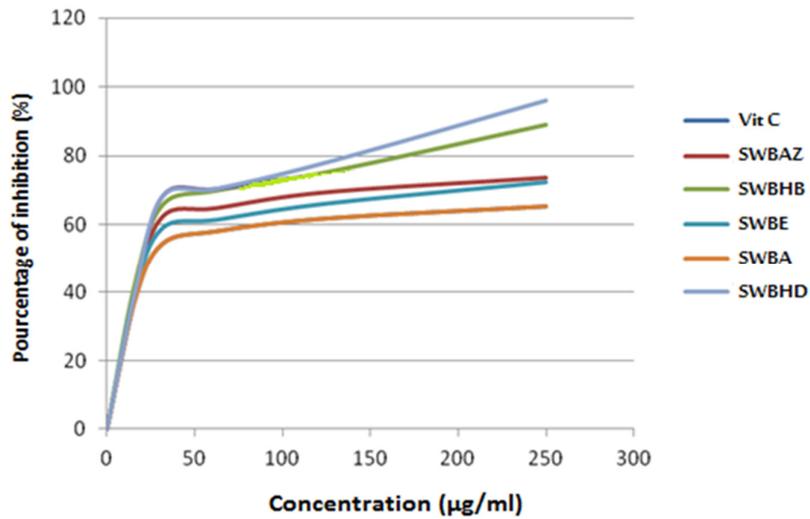


Figure 2. Antioxidant activity of different varieties of soft wheat bran
The different varieties of soft wheat bran samples: SWBHD: Soft wheat Bran HD; SWBA: Soft wheat Bran Anzar; SWB E: Soft wheat Bran Eriad; SWB AZ: Soft wheat Bran Azzouz; SWB HB: Soft wheat Bran Habbour

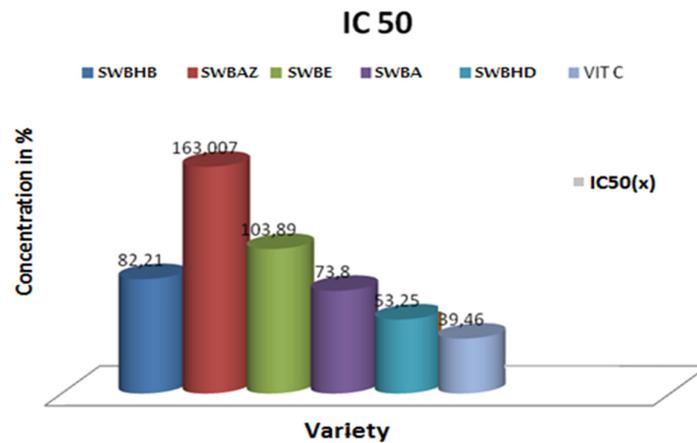


Figure 3. Inhibition concentration of different varieties
The different varieties of soft wheat bran samples: The different varieties of soft wheat bran samples: SWBHD: Soft wheat Bran HD; SWBA: Soft wheat Bran Anzar; SWB E: Soft wheat Bran Eriad; SWB AZ: Soft wheat Bran Azzouz; SWB HB: Soft wheat Bran Habbour

Conclusions

Nowadays, cereals in general, wheat in particular, constitutes the main basis of the diet for the Algerian consumer. The bran, wheat, are the envelope that surrounds the grain of the cereal to protect it, and are a rich source of a set of micronutrients and natural bioactive compounds that have the potential to exert beneficial health effects. The wheat bran component is encouraging the use of wheat bran as an ingredient of functional foods. After an overview of the composition function, and bioavailability of wheat by phenolics compounds. Finally, this relatively high intraspecific variation among genotypes within species, cellulose, sodium and

potassium, total phenolic content, flavonoids, provides a foundation for those interested in using or improving modern wheat varieties for their health benefits.

Authors Contribution

All authors contributed to the review and the editing of the article. MS wrote the manuscript and supervised in final reviewer the manuscript; SS, MN and LL participated in the experiment of this study; ZM in reviewer of the manuscript; MH supervised data analysis; BR for vegetal materials, CFZ designed for English grammar correction, BM designed and conducted the research; All authors read and approved the final version of the manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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