Journal homepage: http://fens.usv.ro/index.php/FENS
Journal of Faculty of Food Engineering,
Ştefan cel Mare University of Suceava, Romania
Volume XXI, Issue 1 - 2022, pag. 82 - 93

BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF PERIPHERAL LAYERS OF SOFT WHEAT GROWN IN ALGERIA DURING SEED FILLING

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Received 31th January 2022, accepted 30th March 2022

Abstract: *Wheat bran is a by-product rich in bioactive compounds. However, little is known about the arrangement, composition and secondary metabolisms of the grain outer layers during grain development. To this end, the objective of this study is to evaluate the variations in cell wall structure and chemical composition of the outer layers during the cellularization phase, the actual filling of the grain and the maturation phase. Morphological and structural aspects were evaluated by scanning electron microscopy; phytochemical study also was carried out: Extracts from the peripheral layers (CP) of wheat grain (cultivar Triticum aestivum HD1220) were analyzed to determine: Total phenols content by Folin-Ciocâlteu method, flavonoids by aluminum trichloride and sodium hydroxide method; Antioxidant power was evaluated by free radical scavenging (DPPH) and iron reduction (FRAP). The results of histological analysis showed that the outer layers become thinner during grain development, the main tissues develop and differentiate synchronously; the phenolic content ranged from 47.58± 3.23 mg GAE/g to 108.67± 10.7 mg GAE/g wheat outer layers, the wheat outer layers contained 6.96±2.61-35.297±8.68 mg EAQ/g flavonoids. The contents of total phenols (TPC) and flavonoids (TFC) increased during development; the antioxidant activity, depended on the developmental phase of the peripheral layers; the antioxidant activity studied for 70% ethanolic extracts was highest in the peripheral layers of mature grains. These results underline that the peripheral layers of wheat bran derived from maternal tissues are constituted by a superposition of different tissues and are considered as an important source of bioactive compounds*

Keywords*: Wheat,Peripheral layers, histology, phenolic compounds, antioxidant activity.*

1. Introduction

Wheat is one of the main cereal crops second only to rice, which is consumed by nearly one third of the world's population. Global wheat (Triticum aestivum) production is estimated to be about 737 million tons [1, 2].It is rich in protein, minerals, vitamins and dietary fiber being considered a nutrient-rich grain [3] .Caryopsis has three main parts: The bran part accounts for 13%~17%, the germ or embryo part accounts for 2%~3%, and the endosperm part accounts for 80%~85% rich in starch.

The peripheral layer of the grain or wheat bran includes the pericarp, the Testa, hyaline and the aleurone layer. A dense cell border aleurone layer envelops the endosperm, peripheral layer contains rich phytochemicals and minerals, including dietary fibre [4], however, wheat bran have been recognized by many researchers possess different antioxidant capacities. Bioactives phytochemicals are not evenly distributed. The germ and bran parts usually contain higher concentrations of bioactive phytochemicals [5] they are becoming more and more popular around the world due to

DOI: https://doi.org/10.4316/fens.2022.009

their related health benefits. The bran part can act as a defense against different chronic diseases, particularly diabetes, which accounts for 3.2 million deaths worldwide every year [6]; studies have shown that adequate intake of cereals polyphenols, including phenolic acids, lignans, flavonoids and carotenoids, can prevent colon cancer, nervous system and cardiovascular diseases[1,7,8]

Grain filling and grain development are essential biological processes in the plant's life cycle, eventually contributing to the final seed yield and quality in all cereal crops [9].Wheat grain development is a complex process involving three successive stages: cellularization (i.e., cell division and differentiation), grain filling, and maturation/desiccation [10]. The first stage comprises cellularization and differentiation, leading to the development of both the embryo and endosperm. The grain filling stage is characterized by the onset of synthesis and accumulation of storage molecules such as starch and gluten proteins. The maturation/desiccation stage is characterized by dehydration of the grain, which gradually enters a quiescent state. The synthesis and accumulation of reserve substances occurs in the cells of the endosperm and the formation of protective structures of various types occurs in the integumentary tissues of the skin [11]. The duration of each stage varies, largely depending upon the genetic background and environmental conditions [12]. Polyphenol compounds are synthesized by plants during their growth [13] mostly esterified to the arabinoxyl side groups of arabinoxylans [6] Phenols are present in cereals in the free and conjugated forms [14].

Most research focuses on specific developmental stages (especially grain filling), with little understanding of biochemical and molecular changes. Herefore, this research aims to determine the structural aspects and concentration in bioactive compounds flavonoids, total phenolic compounds and the antioxidant activity in wheat outer layers during grain filling.

2. Materials and methods

Developing Grain Stages

Samples of peripheral layers were fixed directly on stubs, coated with gold on the sample holder. Digital images of topographical features of the samples were collected using a JSM 5800 scanning electron microscope (JEOL, Tokyo, Japan) under the high vacuum/secondary electron imaging mode at an accelerating voltage of 10 kV and instrumental magnification 900×.

Plant materials

For this study, peripheral layers (PL) were isolated from grains of soft wheat cv HD1220 .Grains were harvested from the Tassala station; seed production and demonstration farm technical institute of field crops (ITGC) Sidi Bel Abbes Algeria ,at several developmental stages from cellularisation to maturation (Fig 1).

The endosperm and the outer layers were separated by hand and they were immediately frozen, and crushed in powder for further biochemical analyses (Table1)

Table 1:

Some characteristics of the phases studied

Samples	Abbreviations	Harvest date	Characteristics
01	PLp1	20.04.2020	Phase 1:Cellularisation Phase
02	PLp2	27.04.2020	Phase 2:Beginning of filling, milk stage
03	PLp3	03.05.2020	Phase 3: Paste Stage
04	PLp4	12.05.2020	Phase 4: Vitreous caryopses
05	PLp5	12.06.2020	Phase5: Maturation phase

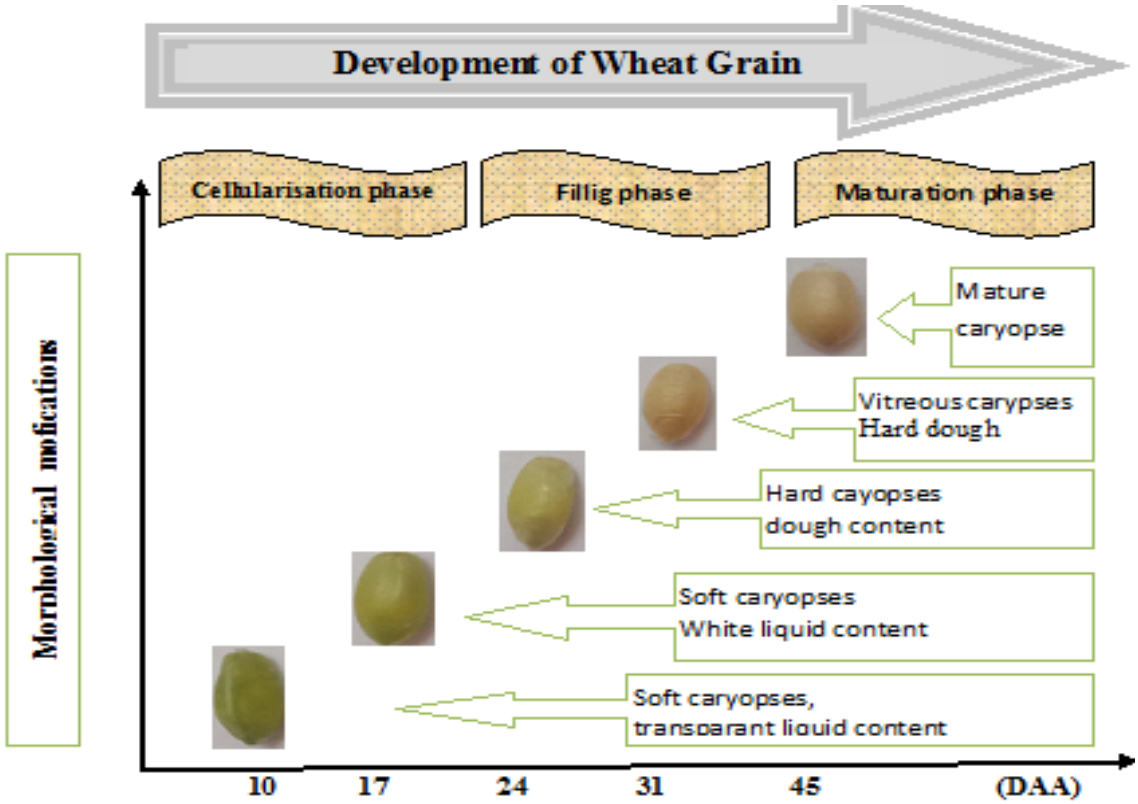


Fig 1. Schematic Trend of wheat grain Caryopses harvested during grain filling.

Extraction of soluble phenolic compounds

In our study, the extraction was performed by maceration in an organic solvent [15]. 5 g of sample were macerated twice in 50 mL ethanol (70%). After stirring for 24 hours to release bound and free phenolic compounds, the mixture was separated by filtration. Then use a rotary evaporator to evaporate the extract to dryness at a temperature of about 45°C. Weighing at this stage can calculate the yield of the extract [16].

Determination of total phenolic compounds TPC

After extraction, total phenolic compounds (TPC) were determined using Folin-Ciocâlteu*'s* phenol reagent [3]. Briefly, 20 µL of extract, 1.58 mL of distilled water, and 100 µL of Folin-Ciocâlteu reagent were measured at 1 Proportional dilution; and 300 µL 10% sodium carbonate, mixed and stored away from the light source. The absorbance was read at a wavelength of 765 nm. The calculation was based on the calibration curve using gallic acid. The concentration of phenols is expressed as µg gallic acid equivalent (GAE)/mg sample. Samples are analyzed in triplicate.

Determination of total flavonoid content (TFC)

The colorimetric method was used to determine the content of flavonoids. In short, 0.5 mL of ethanol extract is diluted with 1 mL of distilled water. Then, add 0.075 mL of 5% $NaNO_2$ solution to the mixture. After 6 min add 0.15 mL of 10% $AlCl_3 \times 6H_2O$ solution and let the mixture

stand for another 5 min. Add half ml of 1 M NaOH and distilled water to 2.5 mL. Mix the solution thoroughly and immediately measure the absorbance of the blank (containing the extraction solvent instead of the sample) at 510 nm. The flavonoids are quantified using a calibration curve obtained by measuring the absorbance of a quercetin smear solution of known concentration, and the results are expressed in microgram equivalents of quercetin per mg of dry extract (mg EQ/g).

Antioxidant Activity

- ***DPPH free radical scavenging activity***

The radical scavenging activity of the peripheral layers extracts was determined using DPPH assay according to [17].A DPPH radical solution with a volume of 0.025 mg mL^{-1} was prepared in methanol, and 1.950 µL of this solution was added to 50 µL of different concentrations of each extract. A negative control was prepared by adding 50 µL of methanol to 1,950 µL of DPPH solution. After incubating for 30 min in the dark at room temperature, use a spectrophotometer to read the absorbance at 517 nm. The positive control is ascorbic acid. The lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The scavenging percentage of DPPH free radicals is calculated as follows:

$$\boldsymbol{DPPH\ (\%) = \frac{(A(control) - A(sample)}{Acontrol * 100}}$$

Among them, DPPH (%) is the reduction percentage of DPPH, A control is the absorbance of the negative control, and A sample is the absorbance of the sample.

The result is expressed as the average of the three measured values obtained for each sample. The percentage reduction of DPPH depends on the concentration curve to graphically reveal the IC50 value that determines the concentration of 50% DPPH free radicals

- **Ferric- reducing antioxidant power (FRAP)**

The ability to reduce Fe^{3+} was estimated by the method described by [18], which is based on the capacity of molecules to convert the oxidation form of iron Fe3+ to its reduced state Fe^{2+}.10 μL of peripheral layers solution at varying concentrations was mixed with 40 μL of 100 mM potassium phosphate buffer (pH 6.6) and 50μL of 1% w/v potassium ferricyanide. The mixture was incubated at 50°C for 20 min, the reaction was terminated by adding 50 μL of trichloroacetic acid (10% w/v), 40 μL of water and 10 μL of 0.1% w/v $FeCl_3$ were then added. The absorbance was then measured at 700 nm. Distilled water was used as negative control. The assay was done in triplicate.

The intensity of the absorbance is proportional to the reducing power of the reaction mixture. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the graph of absorbance at 700 nm against peripheral layers concentration.

3. Results and discussion

Developing Grain Stages

Outer layers play key roles during grain development. Images of morphological changes in the peripheral layers of soft wheat grain of cultivar HD1220 during grain filling observed under a scanning electron microscope (20 μm scale) are shown in Figure 2.

The substructures of peripheral layers are: the pericarp; the testa, the aleurone layer, the nucellus epidermis. The aleurone layer is the innermost layer attached to the pericarp tissue together with the hyaline layer and the testa layer [19].

Outer layers of developing endosperm have been studied in detail [20,21] ;

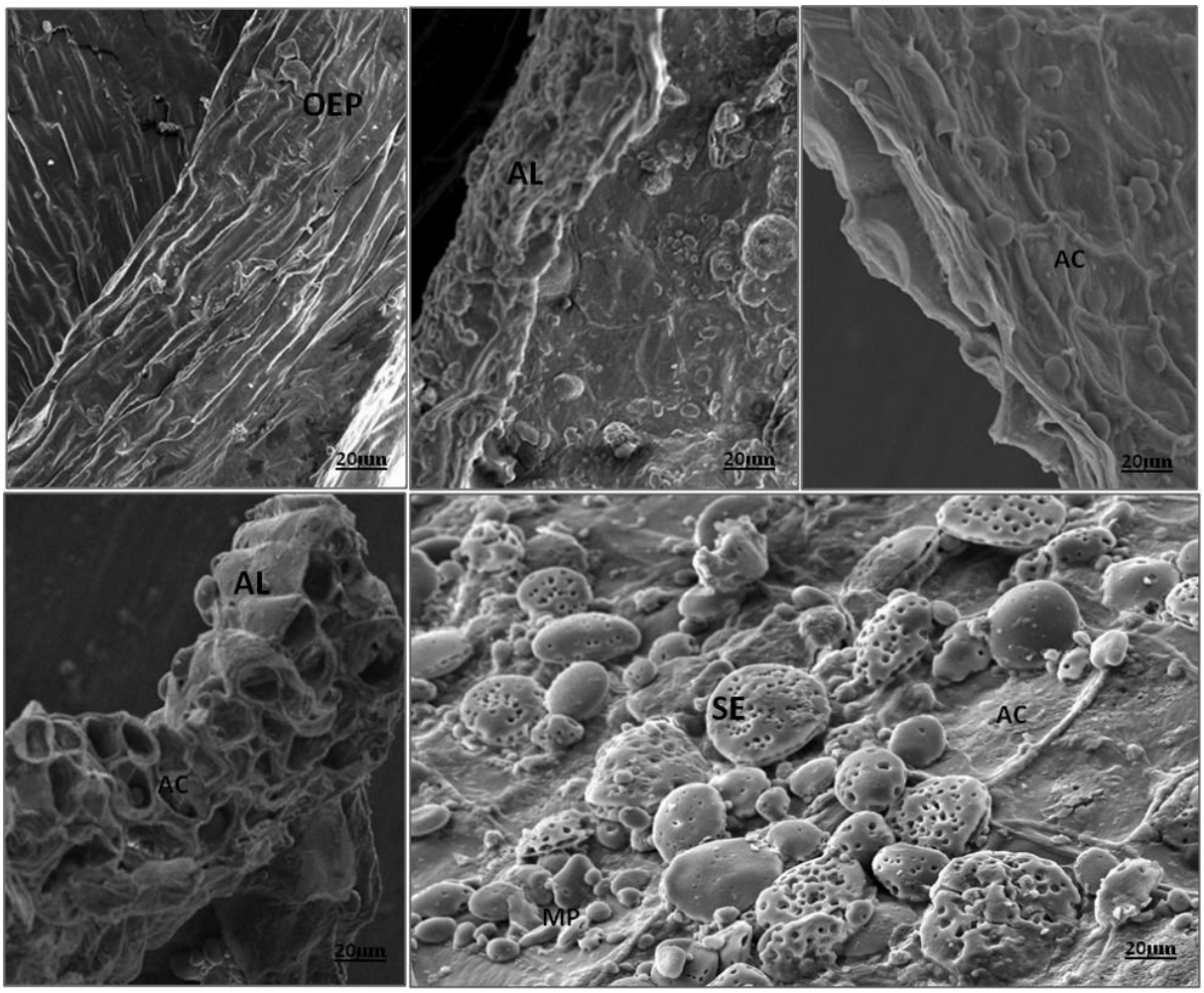


Fig 2. Scanning electron microscopy graph of the peripheral layers and starchy endosperm of soft wheat kernel of cultivar HD1220 during grain filling (900 ×). AL: Aleurone Layer. SE: Starchy Endosperm. OEP: Outer Epidermis of Pericarp. MP: Matrix Proteins. AC: Aleurone Cell

This study also allowed us to highlight the aggregation of starchy granules. All the main tissues (maternal and daughter) grow and differentiate synchronously (coordinately) until the kernel is fully matured (filling) [12]. Observing the first phase PLp1: developing grain mainly undergoes active cell division and differentiation, the cellularization stage is almost finished ,pericarp constitute most of the grain volume, Aleurone cells are not yet differentiated, followed by beginning of grain filling at 17DAA the milk stage' the image shows the pericarp in continuous dark band, which wrap around the caryopses, starch granules begin to form on the inner side of the outer layers of the wheat caryopses, the maternal tissues gradually degenerate, resulting in a decrease in the thickness of the outer layers [22], The aleurone layer becomes recognizable [23].At 24 DAA, corresponded to the beginning of starch accumulation, image reflect larger

polymorphic structures outer layers gradually thicken, Such a modification usually happens due to the deposition of lignin [12].The aleurone cells form unicellular tissue layer surrounding the endosperm [23]. [24] Reported that wheat aleurone is formed from surface endosperm cells and contains densely granular cytoplasms due to the accumulation of aleurone grains. Regarding the starchy endosperm starchy granules are easily observed, regularly distributed and gradually increased in size; At 31 DAA, the starchy granules showed holes in surface due to the dehydration and manipulation of the sample during the section preparation.

At maturity, the peripheral layers derived from the maternal tissues are well differentiated and made up of a superposition of different tissues; from outside to the inner surface, it contains horizontal cells, tubular cells, and two continuous uniform film-like. Structure corresponding respectively to the inner pericarp, the testa, and the nucellus epidermis and inner strip is the aleuron layer.

Results of photochemical analyzes

Yield Extraction

Extraction is a very important step in the separation, identification and use of phenolic compounds. The extraction method depends on the extraction rate of phenolic compounds [25]. It allows us to calculate the yield of each water/ethanol extract. The yield determined relative to 5 g of dried and ground plant material is expressed as a percentage. The results obtained are shown in the (Table 2)

Table 2.

Yield Extraction, concentration of phenolic compounds in mg (EAG)/g and total flavonoid compound mg EAQ/g in peripheral layers during grain filling.

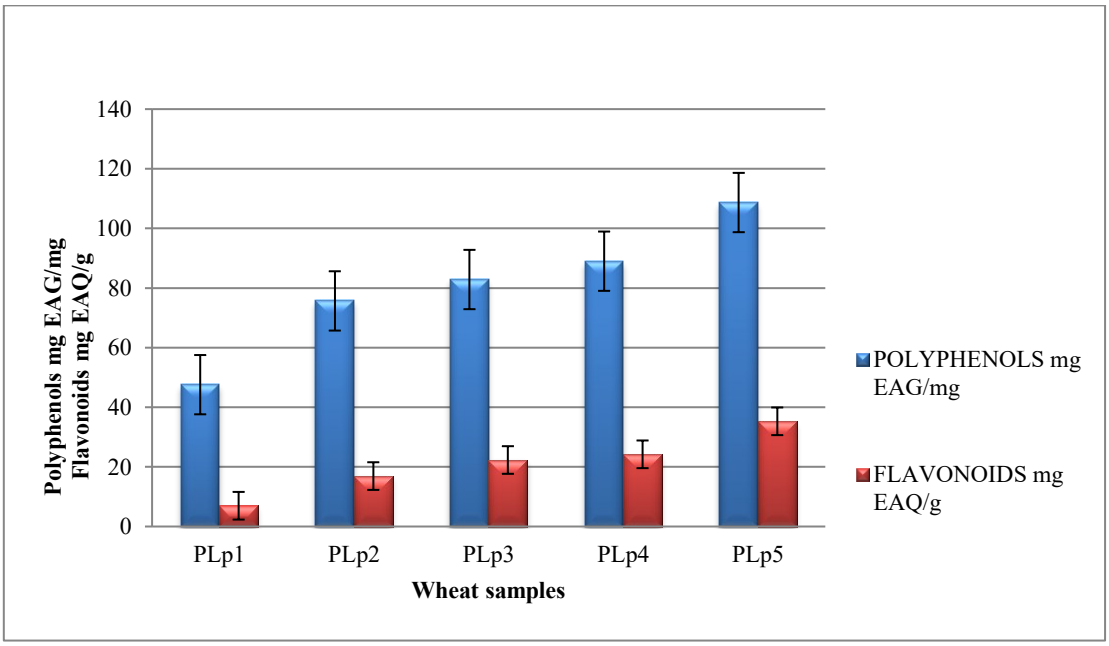
Samples	Yield in %	TPC mg (EAG)/g	TFC mg EAQ/g
PLp1	30.7	47.58± 3.23	6.96±2.61
PLp2	43.91	77.86± 7.12	16.92±5.94
PLp3	33.04	82.84± 2.38	24.643±1.707
PLp4	33.4	88.99 ± 5.27	22.553±2.03
PLp5	22.74	108.67± 10.7	35.297±8.682

TPC: Total Phenolic Compound, **TFC**: Total Flavonoid Compound, **EAG**:Acid Gallic Equivalent, **EAQ**: Quercetin Acid Equivalent. **PLp1**: Peripheral Layer phase 1, **PLp2**: Peripheral Layer phase 2, **PLp3**: Peripheral Layer phase 3, **PLp4**: Peripheral Layer phase 4, **PLp5**: Peripheral Layer phase 5

Results of Total Phenolic Compounds (TPC)

Polyphenolic compounds are secondary metabolites synthesized by plants during its growth and are produced mostly in response to stress [14]. The total phenolics content of PL extracts was determined by Folin-Ciocâlteu assay and expressed in terms of microgram of gallic acid equivalent GAE/mg [17]. Using the standard curve of gallic acid (R^2 = 0.997), results obtained from the extracts of the peripheral layers (PL) analyzed (Fig 01) reveal that PL extracts of mature grains is the richest in phenolic compounds with 108,67± 10,7 mg (EAG)/g followed by PLp4 secondly with a content of 88,99 ± 5,27mg (EAG)/g, PLp3; PLp2 extracts with a content of 82,84± 2,38 mg (EAG) / g 77,86± 7,12 respectively and finally PLp1 extract registers the lowest content with 47,58± 3,23 mg (EAG) /g (Table 2). At early developmental stages cellularisation stage polyphonols register the lowest content; outer layers constitute most of the grain volume and weight during this phase the pericarp contains chloroplasts

rich on chlorophyll indicating that polyphenols content was active in early grain filling stage. The total phenolics content of PL extracts demonstrated a close content between PLp3 with 82,84± 2,38 mg(EAG)/g content and PLp4 of 88,99 ± 5,27 mg(EAG)/g suggesting little differences in their metabolite level coincide with the accumulation of starch reserves in the endosperm. In contrast, PL extracts of mature grains register the highest content in phenolic compounds with 108, 67± 10, 7 mg (EAG)/g.



PLp1: Peripheral Layer phase 1, **PLp2**: Peripheral Layer phase 2, **PLp3**: Peripheral Layer phase 3, **PLp4**: Peripheral Layer phase 4, **PLp5**: Peripheral Layer phase 5

Fig 3. Polyphenol and Flavonoid content of wheat peripheral layers samples.

Previous work by [26] reported that the composition of grains is affected by the environmental and climatic conditions of crop growth, especially the temperature and water supply. The presence of the phenolics compounds in wheat bran is mainly covalently cross-linked with cell wall polymers [27]. Furthermore [28] reported that the accumulation of polyphenolic compounds and the biosynthesis of flavonoids in wheat involve the expression of genes whose expression could be related to drought that in accordance with [6] who reported that the content of phenolic compounds depends on genetic components, as well as genetic interactions with the environment.

The differentiation of the inner layer of the inner integument is associated with the synthesis and deposition of phenolic compounds, which gradually accumulate in the cytoplasm of these cells [12]. It is difficult to compare the results obtained in this study to those of previous studies because of the current study is the first investigation monitoring the antioxidant content and the antioxidant activity during grain development although previous work by [29] showed that the bran/germ fraction contributes 83% of the total phenolic content of the whole meal flour. Actually, the total phenolic content of bran/germ fractions is 15- to 18-fold higher than that of respective endosperm fractions and the

starchy endosperm contributes only 17% of the total phenolic content.

Results of total flavonoid content (TFC)

Flavonoids, a class of low-weight phenolic compounds, exert important role in plants, such as UV protection, as a defense pathogens and pests, as microbial signals, as auxins transport regulation and pigmentation [30].Determined by the method used by Tlili et al., 2015 flavonoid content for PL extracts was reported in mg equivalent quercetin per g of dry extract (R^2 = 0.9921) .The flavonoid levels are expressed in Table2.

Flavonoid concentration is 6,96±2,61 mg EAQ/g (PLp1) , 16,92±5,94 mg EAQ/g (PLp2), 24,64±1,707 mg EAQ/g (PLp3), 22;553±2,03 mg EAQ/g (PLp4), 35,297±8,682 mg EAQ/g (PLp5).

The results demonstrated that flavonoids concentrations ranging from 6, 96±2, 61 mg EAQ/g to 35,297±8,682 mg EAQ/g. It can be seen, from the results, that the lowest rate of flavonoids was found in pheripheral layers phase 1 with a rate of 6, 96±2, 61 mg EAQ/g at increasing concentrations during wheat grain development. Mature peripheral layer registered the highest rate oh flavonoids content with a rate of 35,297±8,682 mg EAQ/g. regarding the flavonoids, the main reason for choosing this class of polyphenols is that flavonoids are the most important polyphenolic class, with more than 5,000 compounds already described (Gomez 2006). Flavonoids are a class of beneficial antioxidant substances of total phenolic compounds and some studies suggest that this class is more effective as antioxidants than Vitamin C[31], [32] also reported that the flavonoids were detected bound to the cell wall of wheat. Our results can be explained in the differences that exist in the accumulation of total flavonoid content during grain filling. In addition, [6] mentioned that The high concentration of flavonoids in wheat bran with ethanol, may be associated with flavonoid aglycone structure, which are easier to extract in ethanol,the response mechanism for flavonoid biosynthesis may be different between wheat cultivars [33].

Determination of antioxidant activity

DPPH radical- scavenging activity

The strong antioxidant activity of flavonoids and phenolic compounds and their ability to scavenge free radicals have received special attention [34]. The DPPH free radical scavenging assay was widely used for evaluating the activity of natural antioxidant, because DPPH radical can form stable molecules pairing with electrons or hydrogen radical. All peripheral layer extracts showed DPPH scavenging activities. The IC50 (50% inhibitory concentration), also called EC50 (Efficient concentration 50), is the concentration of the sample tested necessary to reduce 50% of the DPPH radical. The IC50 are calculated graphically by percentages of inhibition as a function of the different concentrations of the extracts tested, a low value of the IC50 indicates a strong antioxidant activity.

The IC50 values obtained are reported in (Fig 4), ranging from 181,924 mg/mL to 345, 6 mg/ml with ascorbic acid used as (reference antioxidant) with IC50=35.326 mg/g. Our results showed the lowest IC50 at early developmental stages with 345,6 mg/mL in PLp1,than IC50:305,197 mg/ml for PLp2; and the last extracts with values of IC50 224,6 mg/mL for PLp3 extract and IC50: 193,83 mg/ml for PLp4, respectively and finally IC50: 181,924 mg/ml for the mature PL extract. Our results revels that our extracts between them have significant antioxidant potential, at increase in chelating activity during grain filling.

The results of this study are in agreement with the work of Luthria et al.,2015 [5] who reported that antioxidant capacity in wheat is associated to its content of polyphenolic compounds, due to the presence of phenolic acids located in the outer layers of the wheat grain .In addition [14] also reported that extracts with a higher content of phenolics possess a higher antioxidant activity. Furthermore [35] reported that genotypes, extraction method, the choice of solvent used and also the dosage methods, the presence of hydroxyl groups as hydrogen donor constitute essential factors of these variations. The presence of antioxidant compounds and the level of antioxidant activity in wheat are significantly affected by the genotype, the growing area [36], and environmental conditions [37]

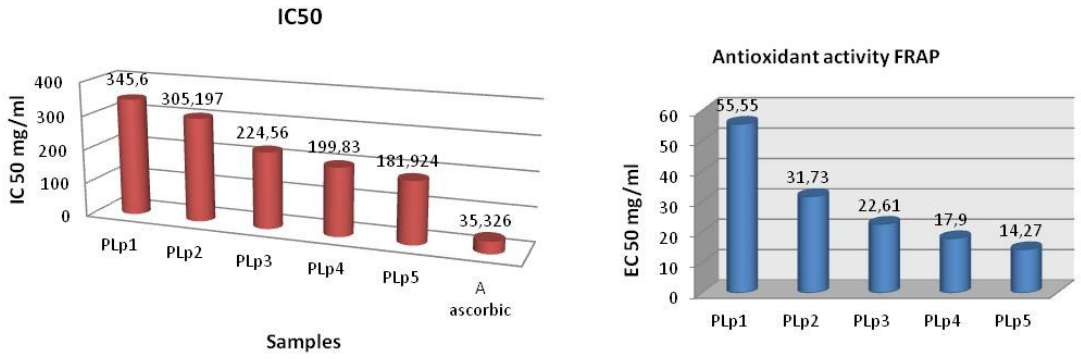


Fig. 4. Histogram of the values of the inhibitory concentrations IC50 in mg / mL and Iron reduction EC50 of the extracts by FRAP assay of the different samples

PLp1: Peripheral Layer phase 1, **PLp2**: Peripheral Layer phase 2, **PLp3**: Peripheral Layer phase 3, **PLp4**: Peripheral Layer phase 4, **PLp5**: Peripheral Layer phase 5

Iron reduction: FRAP (Ferric reducing antioxidant power)

It is a measure of the ability of the substances in our extract to reduce ferric iron Fe^{3+} to divalent iron Fe^{2+}. This is a fast, simple and reproducible technique [38]. The reducing ability of a compound can be used as an important indicator of its potential antioxidant activity [39]. Previous work has shown that there is a direct relationship between antioxidant activity and the reducing ability of some plants [40]. In our work, we tested different PL extracts from HD 1220 wheat varieties through the FRAP method, with ascorbic acid used as (reference antioxidant) and the results obtained allowed us to draw a curve for each extract. We noticed that the iron reduction capacity is proportional to the increase in sample concentration [41, 42]. The results obtained show that the capacity of our extracts to reduce iron is lower than that of ascorbic acid for the five samples, but it is variable between the extract of the peripheral layers studied,the comparison of the antioxidant power of our extracts between them reveals that the peripheral layer of mature grains is the most active whereas the extract of PLp1 sample had the lowest capacity iron reduction. If we classify our extracts according to the reduction power of iron compared to ascorbic acid, we will obtain the following order: ascorbic acid> PLp5 > PLp4> PLp3> PLp2> PLp1.This indicates iron reduction capacity increased during grain

development due the fact that bran cumulate during its development phenolic compounds directly associated with antioxidant activity [43], that a high concentration in phenolic compounds of each extract increases its reducing power of iron. These results were confirmed by the DPPH free radical scavenging assay; Moreover [6] reported that the variation of antioxidant activity besides depending on the polarity of the phenolic compounds and flavonoids. It also depends on selectivity and action mechanism of radical used [32].

4. Conclusion

The evaluation of bioactive compounds revealed the accumulation of a considerable amount of polyphenols and flavonoids, qualitatively and quantitatively during the filling of the wheat grain. It was also demonstrated that the accumulation of bioactive compounds, were affected by several factors, such as genetic components, environmental conditions and agronomic practices. It was established that antioxidant activity was found to increase throughout grain development especially in the final phase; soft wheat bran is an important source of antioxidants influenced by environmental factors characterized by grain cultivation and the varietal effect of wheat grain.

5. Acknowledgments

The authors acknowledge the University of Sidi Bel Abbes, Department of biology for the financial support of this study.

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