Advances in Bioresearch
Adv. Biores., Vol 11 (2) March 2020: 40-50
©2020 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.11.2.4050

Advances in Bioresearch

ORIGINAL ARTICLE

Comparative study of antioxidant and anticholinesterase activity of unfermented and fermented wheat and couscous from fermented wheat «El hammoum», traditional Algerian product

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ABSTRACT
Fermentation can be used as an alternative to genetic engineering in improving the nutritive values (such as amino acids, vitamins, minerals, etc.), functional (temperature), chemical (pH, amylase, total starch etc.) and bioactive properties of grains. In Algeria, "El Hammoum", durum wheat fermented, from storage in underground silos (Matmor) is one of the most popular diets used extensively in some rural areas. It is considered as an important nutritional and antioxidant source. The antioxidant activity of "El Hammoum" might be of particular interest to medical researchers and needs further attention regarding its implication in many physiological processes. Eight methods, based on different principles, were used to evaluate antioxidant activities: including DPPH, FRAP, CUPRAC, metal chelating, β-carotene/ linolenic acid and ABTS. Furthermore, anticholinesterase activity was determined against acetyl cholinesterase (AChE) and Butyrylcholinestérase (BChE) enzymes using the Ellman method. The results showed high antioxidant potentials in the "El Hammoum" fermented wheat, couscous from fermented wheat, than unfermented wheat for the various antioxidants assays performed. The order of antioxidant activity of extracts based on DPPH method was: FWH = CH > UFW, which is similar and consistent with, ABTS, phenanthroline, Metal chelating activity, FRAP, DMSO. We concluded through our studies, fermentation increases the health benefits by increasing its free radical scavenging abilities higher. Accordingly the antioxidant activity is correlated to the phenolic content of the extracts. The best inhibitory activity on anticholinesterase was exhibited by the fermented wheat, couscous from fermented wheat in vitro. Finally, In light of these findings, it can be concluded that the fermented wheat and couscous from fermented wheat have shown moderate antioxidant and enzyme inhibitory activities and can be used as a functional food
Keywords; *Matmor, traditional fermented wheat, natural fermentation, antioxidant, anticholinesterase activities*

Received 16.01.2020 Revised 22.02.2020 Accepted 02.03.2020

How to cite this article:
Mokhtari S, Bensouici C, Saidi D, Kheroua O. Comparative study of antioxidant and anticholinesterase activity of unfermented and fermented wheat and couscous from fermented wheat «El hammoum», traditional Algerian product. Adv. Biores., Vol 11 (2) March 2020: 40-50

INTRODUCTION

Recently, there has been a growing interest in research with regard to the role of cereals derived antioxidants in food and human health [11 - 31]. Nevertheless, phytochemicals and antioxidants in whole grains of cereals have not received as much attention as those in fruits and vegetables [15]. The imbalance in production of antioxidants by cells leads to the overproduction of free radicals, which are implicated in inducing oxidative damage to biological molecules such as oxidizable lipids, proteins and DNA leads to a many age-related diseases as cardiovascular disorders, cancer, neurodegenerative diseases as Alzheimer [2-9]. Many researchers have led to the discovery of an important number of naturally-occurring compounds of food-derived secondary metabolites with antioxidant and anti-AChE activities. Especial, "El-Hammoum" has been recognized as a healthy food for a long time that may synergistically contribute to their protective effects; the popularity of "El Hammoum "as a part of the human diet has increased because of reports describing the beneficial nutritional properties of fibres,

proteins, carbohydrates, lactic acid bacteria [22]. Fermentation of grains is of emerging interest, which may significantly enhance the nutritional, functional and bioactive content of grains. The fermented wheat is generally used for preparing couscous. The couscous is a typical Algerian product, prepared according to a traditional process. "El Hammoum" is still not fully explored as a source of metabolites with therapeutic potential for human diseases. Few papers have described the fermented wheat bioactivity. However, the objectives of this study were:

- To investigate antioxidant activities using different tests in vitro.
- To quantify the phenolic and flavonoid contents present in FWH, UFW and CH.
- To estimate the Anticholinesterase capacities.

MATERIAL AND METHODS

Samples Source

The fermented wheat (Figure 1D) for the experimental production of couscous "El Hammoum", was the origin of "Matmor" (Figure 1C) belonging to a farmer located in the region of Ferraguig (Mascara, Algeria) with Latitude 35.5331 (35°31′59″) North, and Longitude 0.155139 (0°9′19″) East. After four years of fermentation, wheat grains were dried in the sun. They are black and brown colors, and with a special aroma. All samples were transferred to the laboratory under refrigeration and stored at 4 °C until their analysis.

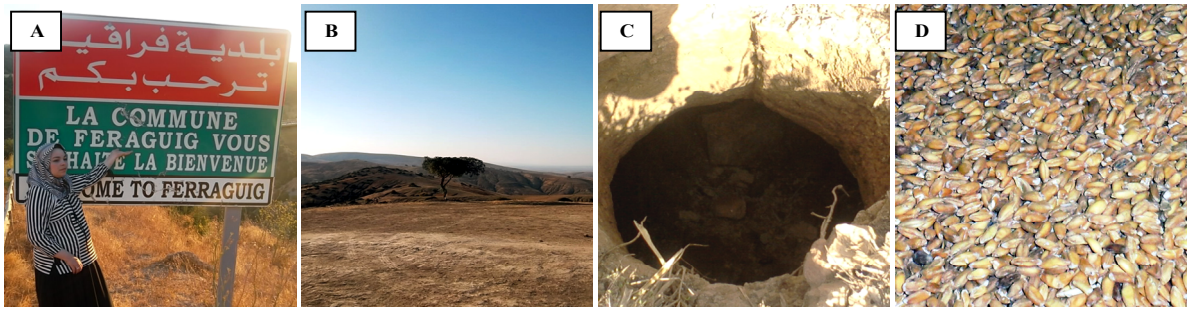

Figure1: Fermented Wheat (D), Opening of the matmor (C) and sampling area (A, B)

Traditional method used for preparing couscous "El Hammoum"

We tried to fabricate couscous from milled products where granulometry distribution differs. Traditional utensils used are shown in figure 2. The steam-cooker is a kitchen utensil, composed of two parts. The lower part is used to boil water, the steam generated is used for cooking couscous, in the upper part called keepsakes. The latter is composed of small holes that allow the rising steam to cook the couscous. Operations of rolling for aggregating semolina are realized in a large wooden dish called "guessâa". Four different sieves named "dekkak", "reffad", "meâaoudi" and "sekkat" are utilized in sieving or sizing operations.

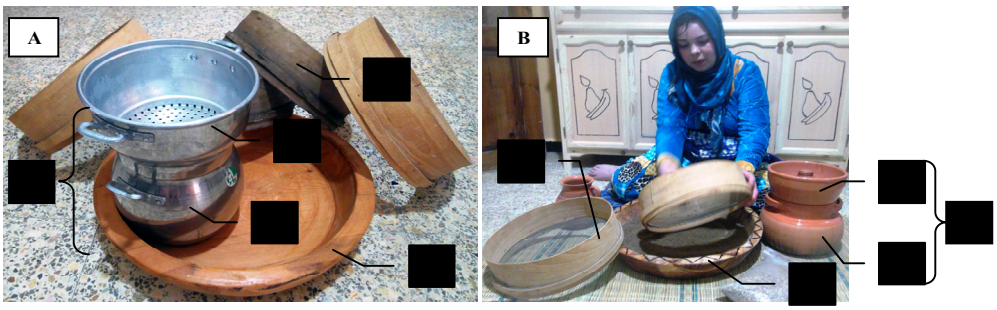

Figure2: Utensils for making couscous "El Hammoum" (A) and traditional method used for preparing couscous "El Hammoum" (B), where a - "gessâa", b - "borma", c - "keskess"; d - "couscoussier", e - sieves named "dekkak", "reffad", "meâaoudi" and "sekkat"

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Figure3: UFW - Unfermented Wheat (A), FWH - Fermented Wheat (B) and CH - Couscous of Fermented Wheat (CH)

Preparation of methanolic extracts

The methanolic extract of samples was obtained by maceration of 30g of powder in 300 ml of methanol (80%) for 24 hours at room temperature with constant stirring. The same powder was extracted two times. To remove the solvent, combined filtrates obtained were concentrated under reduced pressure and dried at 45°C.

Quantitative determination of chemical constituents

Total phenolic contents

Total phenolics in crude extracts of FWH, CH and UFW were determined by the Folin–Ciocalteu method (colorimetric method) [28], which is considered the best method for total phenolics. Gallic acid solution at various concentrations was used for calibration. The Total phenolics of samples were expressed as Gallic acid equivalents (µg EAG/ mg extract) by means of a dose response curve for a Gallic acid (y=0.003x+0.104 (R2=0.997)).

Total flavonoid contents

The total flavonoid content in the extract was determined spectrophotometrically based on flavonoid aluminum complication. After 40 min at room temperature, the absorbance was determined at 415. Quercetin was used as a standard. The concentrations of flavonoid compounds were calculated according to following equation that was obtained from the standard quercetin graph: flavonoid was reported in µg equivalent quercetin /mg (R^2 = 0.997).

***In vitro* evaluation of antioxidant assays**

DPPH free radical scavenging assay

The free radical-scavenging activity was determined spectrophotometrically by the DPPH assay [5]. A solution of 40 µl of the sample (extracts, pure compounds) at various concentrations was added to 160 µl of the methanolic solution of DPPH (0.1 mM). Blanks were prepared using the solvent in addition to the DPPH reagent. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 Musing a microplate. Alpha-Tocopherol was used as standard. The scavenging capability of DPPH radical was calculated using the following equation. The results were given as IC50 values (µg/ml) corresponding the concentration of 50% inhibition:

% scavenging effect = [(A Blanc – A Sample)/A Blanc] x 100

ABTS free radical scavenging activity assay

The ABTS decolorization assay was determined by the method described previously [25]. Then, to 40 µl of the sample solution in methanol at different concentrations was added 160 µl of ABTS solution. The mixture was left at ambient temperature for 10 min and then the absorbance was measured at 734 using a 96-well microplate reader. While Butylated Hydroxyanisole (BHA) was used as a standard. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (methanol). The scavenging capability of ABTS was calculated using the following equation:

ABTs scavenging effect % = [(A Blanc – A Sample)/A Blanc] x 100

Cupric reducing antioxidant capacity assay

The cupric reducing antioxidant capacity (CUPRAC) was determined according to the CUPRAC method [23]. To 50 µl of Cu (II) solution (10 mm) was added 50 µl of neocuproine solution (7.5 mm) and 60 µl of NH_4Ac buffer (1 M, pH 7.0) solution. To the above mixture was added, 40 µl of the sample solutions (extracts and pure compound) at different concentrations. After 60 min, the absorbance at 450 was recorded against a reagent blank using a 96-well microplate reader. The results were given as $A_{0.5}$ (µg/ml) corresponding the concentration indicating 0.5000 absorbent intensity. BHA and butylated hydroxytoluene (BHT) was used as a standard.

Ferric reducing antioxidant power (FRAP)

The reducing power was measured by small changes according to [29]. Briefly, a solution (10 µl) of the sample at various concentrations was mixed with 40 µl of phosphate buffer (0.2 M, pH 6.6) and 50 µl of

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potassium ferricyanide (10 mg/ml). The obtained solution was incubated at 50C° for 20 min. Then, the solution was mixed with 50 µl of trichloroacetic acid solution (100 mg/ml), 10 µl of ferric chloride solution (1.0 g/l), and finally diluted with 40 µl of distilled water. The absorbance was measured at 700. The results were given as A0.5 (µg/ml). The increase of reducing power by the extract was calculated as follows. Where, A test and A Blanc are absorbance of sample and blank solutions, respectively.

$$\% \text{ reducing power} = [(A \text{ test}/A \text{ Blanc})-1] \times 100$$

β-carotene bleaching assay

The assay was determined according to the [7] method. A stock solution of β-carotene/ linolenic acid was initially prepared by mixing a solution of β-carotene (5 mg) in chloroform (1 ml) with linolenic acid (25 µl) and Tween 40 emulsifier (200 mg). The mixture was evaporated under vacuum to remove chloroform. Then, 100 ml of water saturated with oxygen was added to the above mixture by agitation. A volume of 40 µl of the samples (in methanol) at different concentrations was mixed with 160 µl of the above mixture. Immediately, the absorbance at zero time (A0) was measured at 470 using a 96-well microplate reader. The plate was incubated at 50°C for 2 h, and then, the absorbance (At) of the mixture was measured again at 470 NM. BHT was used as standard. Methanol was used as the blank solution. The bleaching rate of carotene was calculated according to the equation: where, A_0 and A_t are absorbent at time zero and t = 120 min, In is the natural logarithm. The antioxidant activity was calculated in terms of percentage inhibition relative to the blank using the equation R=ln (A0 /At)/t:

$$\% \text{ Antioxidant activity} = [(A \text{ test}/A \text{ Blanc})-1] \times 100$$

Metal chelating activity assay

The metal chelating activity by the foreign-Fe^2 complexation assay measured spectrophotometrically [8] with slight modifications. The extract solution dissolved in methanol in different concentrations, were added to 40 µL 0.2 mm Fe^{+2} +40 µl MeOH. The reaction was initiated after addition of 80µL 0.5mm frame. The mixture was shaken then left at room temperature for 10min. At the equilibrium, the absorbance was measured at 593 nm. The activity was calculated by the use of the following equation. The results were given as IC50 values (µg/ml) corresponding the concentration indicating 50% inhibition: metal chelating activity

$$\% = [((R_{\text{blanc}} - R_{\text{Sample}}) \times \text{blanc})/R] \times 100$$

Scavenging of superoxide radical by alkaline DMSO assay

The superoxide radical scavenging ability was measured using alkaline DMSO according to [26]. Method adapted to 96-well microplates. The reaction mixture consisted of 40 µL of extracts at varying concentrations (0-4mg/mL in DMSO), 130 µL of alkaline DMSO (1 ml DMSO containing, 5 mm NaOH in 0.1 ml water) and 30 µL NBT (1 mg/ml in DMSO). The mixture was incubated at 25 °C for 5 min, and absorbance was measured at 560 NM. Ascorbic acid was used as positive control. The scavenging activity is determined using the equation:

$$\text{Scavenging activity} = [A \text{ Sample} - A \text{ Control}] \times 100/ A \text{ Sample}$$

Determination of the Anticholinesterase activity assay

Acetyl cholinesterase (AChE) and BChE inhibitory activities were measured by slightly modifying the spectrophotomertic method [13]. Ache from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. For the measurement of the activity 5, 50-Dithiobis (2- nitrobenzoic) (DTNB) acid was used. Briefly, 150 µL of 0.1 M sodium phosphate buffer (pH 8.0), 20µL of AChE (or BuChE) solution and 10 µL of extract solution at different concentrations (500 µg-7, 81 µg) were added in a 96-well microplate. After an incubation period of 15mn at 25°C, the reaction was then initiated by the addition of 10µL of DTNB and 10 µL of substrate (ACHI or BCHC). The results of the reaction were monitored spectrophotometrically at 412 nm. Galanthamine was used as positive control. The results were given as IC50 values (µg/ml). Percent AChE enzyme inhibition was calculated using following formular:

$$\% \text{ Inhibition} = [A \text{ Control} - A \text{ Sample}] \times 100/ A \text{ Control}$$

Statistical analysis

The concentration giving 50% inhibition (IC50) was calculated with Microsoft excel 2013. Statistical significance was determined by one way analysis of variance (ANOVA) using RSTAT. Significant differences between means were determined by Tukey method. $P < 0.05$ was considered as significantly different.

RESULTS AND DISCUSSION

Characteristics of methanolic extract

Characteristics of methanolic extracts of FWH, CH and UFW are shown in table 1.

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Quantification of polyphenols and flavonoïds

The results of the Phenolic and flavonoid contents are shown in table 3 and figures 4, 5. The extract of fermented wheat is characterized by the presence of considerable amount of phenolic and flavonoïds compounds whith a very highly significant difference following the order FWH=CH>UFW. The content of phenolic compound was increased after fermentation [10 - 24]. The results obtained concordant with the studies conducted by [3-14-17-21] that confirmed the increase in polyphenol content during fermentation. The level of polyphenols present in our extracts is very important this could be useful to consider the study of biological activities.

DPPH free radical scavenging assay

The fermented wheat extract had the highest antioxidant properties with very highest significant difference P <0.0001 at a concentration between (250 µg and 500 µg). But the unfermented wheat showed 50% inhibition IC50 at a concentration between (1000 µg and 2000 µg) (table 2). The levels of inhibition of DPPH recorded in the presence of the various concentrations of samples are lower than observed with standard BHA. BHT and α-Tocopherol (6.14±0.41, 12.99±0.41 & 13.02±5.17 respectively). IC50 registered in our assay was lower and with potent antioxidant activities compared to previous studies by [20]. Due to the fact that the antioxidant power depends on the chosen method on the concentration and on the nature of physicochemical properties of study antioxidants [1, 4].

ABTS free radical scavenging activity assay

Fermented wheat has a good activity with a very highly significant difference. But the levels of inhibition of ABTS recorded in the presence of the various concentrations extracts of FWH CH UFW are lower than observed with standard BHA, BHT (IC 50 1.81±0.10 & 1.29±0.30 respectively). These findings showed that FWH has a higher inhibitory activity compared to results of medicinal plants *Cistus salviifolius L* and *Cistus monspeliensis* use as alternative medicine (IC50value: 253.83±0.72 and 259.50±0.43µg/mL) [27]. Re et al. (1999) [25] reported an increase in the ABTS scavenging activity with the increase in the concentration of extracts.

Phenanthroline

Similarly, fermented wheat extract exhibited the best performance P<0.0001. All extracts were less effective than the synthetic antioxidant BHT (IC50 = 12.99±0.41).

Scavenging of superoxide radical by alkaline DMSO assay

FWH showed very highly inhibition (P<0.0001). Antioxidant activity DMSO increased with increasing amount of extracts in the reaction. All the concentrations tested of the extracts showed lower antioxidant activity than α-Tocopherol and Ascorbic acid 31.52 ± 2.22, 7.59±1.16 µg/ml.

Cupric reducing antioxidant capacity assay

Activity absorbance increased linearly with the increasing amount of extracts. The UFW extract exhibited the highest activity (P<0.0001) among the extracts. followed by FWH. However none of the extracts exhibited higher activity than those of antioxidant standards. Whereas absorbance 0.5 of BHA and BHT was 5.35±0.71 & 8.97±3.94 respectively. These finding showed that FWH has a higher inhibitory activity compared to results showed by [20] $A_{0.50}$ value: 529.79 ± 48.65 µg/ml (see Table 2).

Ferric reducing antioxidant power (FRAP)

In the presence of differents concentrations of the extract. the extract of fermented wheat showed a potent reducing ability with a very highly significant difference (P<0.001) (see table 2). Extract of unfermented wheat did not actat concentration 2000 µg/ml. These results were weak compared to the standards used in this study (ascorbic acid, alpha- Tocopherol and tannic acid) which showed concentrations corresponding to 0.5 absorbance equal to 6.77±1.15, 34.93 and 5.39±0.91 µg/ml respectively. These findings showed that WFH has a higher inhibitory activity compared to the results showed by [27].

β-carotene bleaching assay

The antioxidant potential of wheat extracts can be evaluated by determining the ability to inhibit β-carotene oxidation. All the concentrations tested of extracts FWH UFW CH showed not active at 2000 µg (see Table 2).

Metal chelating activity assay

The FWH extract also showed the highest metal chelating activity with (P<0.001) (see Table 3). The order of antioxidant activity of extracts based on the metal chelating activity method was FWH>CH>UFW which is similar and consistent with DPPH, ABTS, Phenanthroline, FRAP, DMSO results and phenolic content. This difference between FWH and UFW due to the fermentation [6-12-19]. Same results obtained with the sprouted wheats by [30] that confirmed the sprouted wheats show higher total phenolic content and antioxidant activity than the germinated wheats as extracted by an aqueous 80% ethanol.

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Anticholinesterase activity assay

Table 4 shows the AChE and BChE inhibitory activities of the extracts. compared with that of galantamine used as a standard drug to treat mild Alzheimer's disease. The FWH extract showed higher AChE and the good BChE inhibitory activities (P<0.001) [16 - 18] say that. The presence of flavonoids in the extract may have caused beach inhibitory activity.

The used methods have different reaction mechanisms. However the eight methods clearly indicated that the studied of fermented wheat possess antioxidant activity with difference statistically significant. In this work we reported the Anticholinesterase activity of "El Hammoum". The inhibitory effect of the fermented wheat extract pointed to the existence of a possible synergistic inters action amongst the components in this extract. It can be said that there is a correlation between antioxidant and AChE inhibitory activities. The fermented wheat El Hammoum especially constitutes a valuable alternative and complement in therapies. The findings obtained in this study support the traditional uses of those fermented wheat in nutritional cultures and health promotion of local Algerian communities. Finally further research is needed toward isolation and identification of active principles present in the different extracts which could possibly be exploited for pharmaceutical use.

Table1; Characteristics of methanolic extract

Methanolic extracts	Aspect	Odors	Color
Fermented wheat El Hammoum	Viscous	Strong and characteristic odors	Brown
Unfermented wheat	Viscous	Pleasant odors	Yellow
Couscous of fermented wheat	Viscous	Strong and characteristic odors	Brown

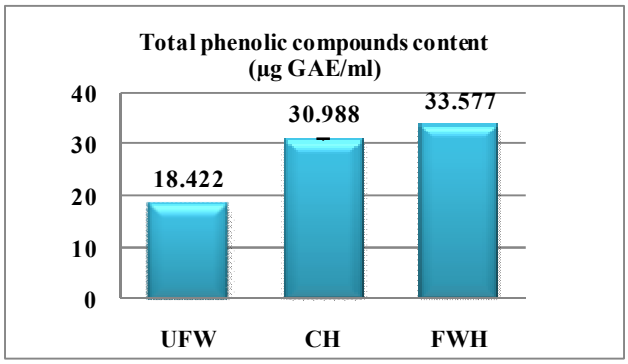

Figure4: Total polyphenol content expressed as gallicacid equivalents (µg GAE/ml) in fermented wheat (FWH), couscous El Hammoum (CH), unfermented wheat (UFW), Each value is represented as mean ±DS (n=3)

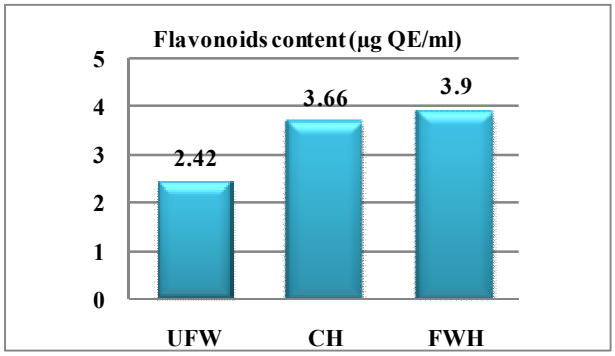

Figure5: Total flavonoïds content expressed as quercetine quivalents (µg QE/ml) in fermented wheat (FWH), couscous El Hammoum (CH), unfermented wheat (UFW), each value is represented as mean ±DS (n=3)

***In vitro* evaluation of antioxidant assays**

Table 2, 3 and figure 6 - 12 shows the antioxidant activity of different concentration extracts of FWH, UFW and CH compared to standards.

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Table 2; Antioxidant activity of FWH, UFW, CH

Compound	DPPH assay (IC 50 µg/mL)[1]	ABTS assay TEAC value (IC 50 µg/mL)[1]	β-carotene bleaching assay	Cupric assay ($A_{0.5}$ µg/mL)[1]	FRAP assay ($A_{0.5}$ µg/mL)[1]
FWH	331.20±0.67 [b]	190.69±0.44 [b]	>2000 µg	366.9±0.85 [b]	459.33±0.57 [a]
UFW	698.57±0.98[a]	377.52±0.38 [a]	>2000 µg	236.33±1.52 [a]	>500 µg
CH	403.35±0.46 [c]	227.75±0.68 [c]	>2000 µg	378.66±0.5 [c]	483.67±0.57[b]
BHA	6.14±0.41	1.81±0.10	1.05±0.03	5.35±0.71	-
BHT	12.99±0.41	1.29±0.30	0.91±0.01	8.97±3.94	-
a-Tocopherol	13.02±5.17	-	-	-	34.93±2.38
Ascorbic acid	-	-	-	-	6.77±1.15
Tannic acid	-	-	-	-	5.39±0.91
EDTA	-	-	-	-	-

[1]IC50 and $A_{0.5}$ values represent the means±SEM of three parallel measurements, - Not tested

Table 3; Antioxidant activity and Total phenolic, flavonoids contents

Compound	DMSO Alkaline assay (IC 50 µg/mL)[1]	Metal chelate assay (IC 50 µg/mL)[1]	phenanthroline assay (IC 50 µg/mL)[1]	Total phenolic content (µg GAE/ml)	Flavonoids content (µg QE/ml)
FWH	328.68±0.61[b]	186.34±0.10 [b]	339.33±0.57 [b]	33.57±0.032 [b]	3.9±0.012 [b]
UFW	448.50±0.93 [a]	256.04±0.80 [a]	484.67±0.57 [a]	18.422±0.024 [a]	2.42±0.002 [a]
CH	388.93±0.70 [c]	207.59±0.37 [c]	346.67±0.57 [c]	30.98±0.050 [b]	3.66±0.001 [b]
BHA	-	-	-	-	-
BHT	-	-	12.99±0.41	-	-
a-Tocopherol	31.52 ± 2.22	-	-	-	-
Ascorbic acid	7.59±1.16	-	-	-	-
Tannic acid	-	-	-	-	-
EDTA	-	3.47±0.35	-	-	

[1]IC50 and $A_{0.5}$ values represent the means±SEM of three parallel measurements

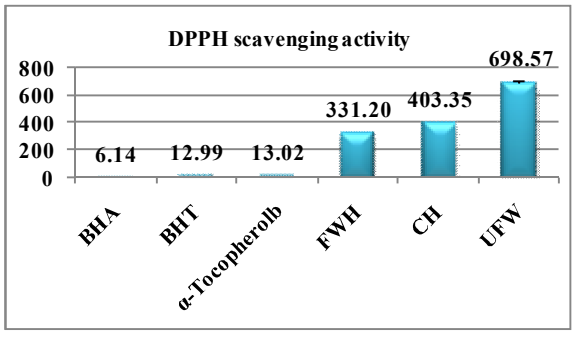

Figure6: Radical scavenging activities of different concentrations of methanol extract and standard antioxidants by free radical DPPH

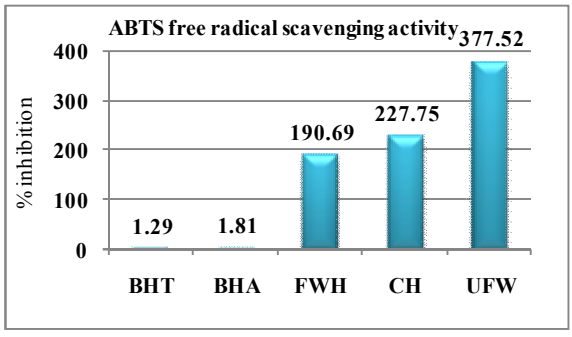

Figure7: Radical scavenging activities of different concentrations of methanol extract and standard antioxidants by ABTS free radical

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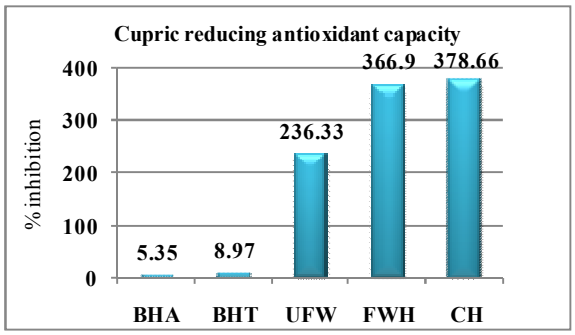

Figure8: $A_{0.5}$ Values expressed in µg/ml of the samples studied sort in ascending order

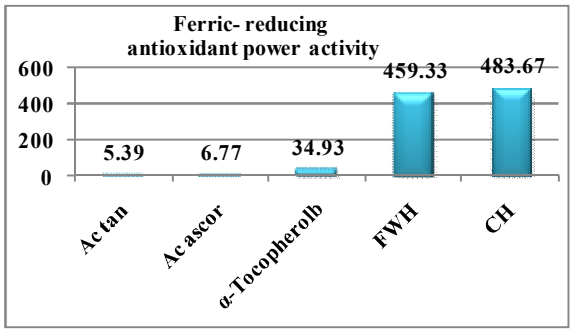

Figure9: $A_{0.5}$ values expressed in µg/ml of the samples studied sort in ascending order

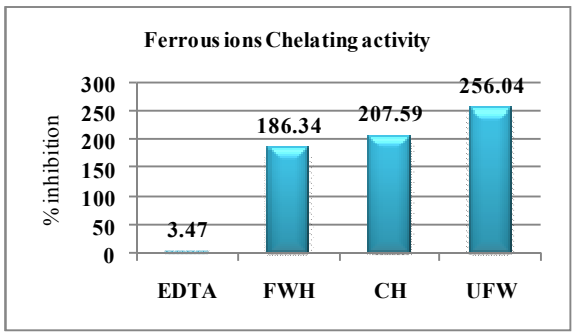

Figure10: IC50 values expressed in µg/ml of the samples studied sort in ascending order

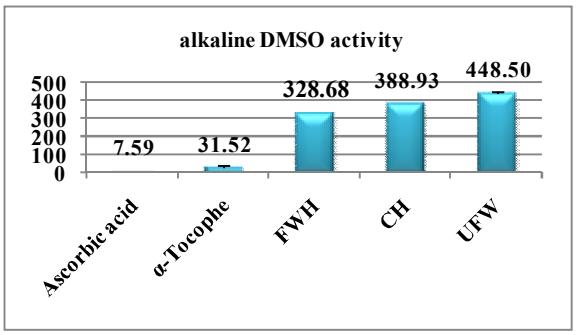

Figure11: IC50 values expressed in µg/ml of the samples studied sort in ascending order

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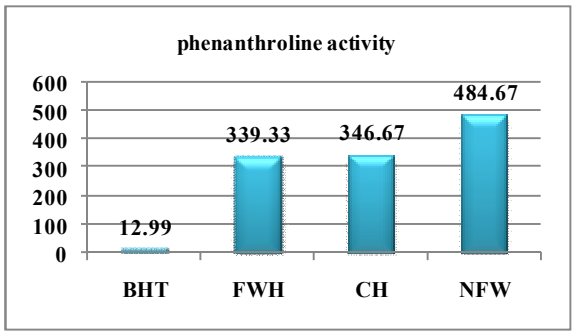

Figure12: IC50 values expressed in µg/ml of the samples studied sort in ascending order

Table 4; Enzyme inhibition activity by FWH, UFW, CH

Compound	AChE (IC50 µg/ml [1])	BChE (IC50 µg/ml [1])
FWH	108.08±0.59[b]	316.06±0.83[b]
UFW	155.42±0.98[a]	369.84±0.68[a]
CH	138.14±0.80[c]	333.79±0.63[c]
Galantamine	6.27±1.15	34.7±51.99

[1]IC50 values expressed are means ± SD of three parallel measurements ($p < 0.05$)

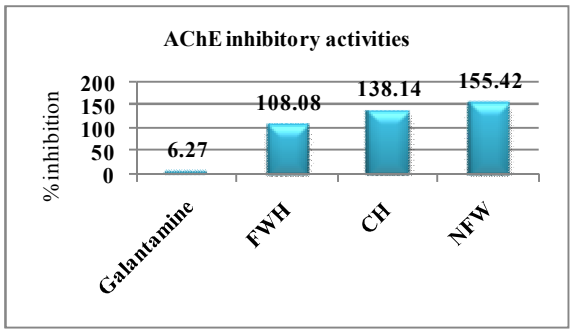

Figure13: IC50 values expressed in µg/ml of the samples studied sort in ascending order

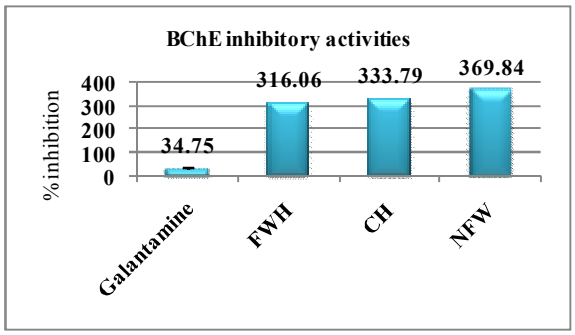

Figure14: IC50 values expressed in µg/ml of the samples studied sort in ascending order

CONCLUSION

The determination of antioxidant capacity has gained a growing interest as a tool for exploring the putative role of antioxidant-rich products in the prevention of degenerative diseases and for the selection of Food with potentially positive health beneits. Our results have proved that fermented wheat and couscous from fermented wheat show high nutritional value and interesting antioxidant properties, which may suggest that fermented wheat secondary metabolites may actas potential therapeutic agent in many pathophysiologic processes where oxidative stress is implicated and a strong contribution to human nutritional therapy. The present study provides the cognition enhancing potential of "El Hammoum" by inhibiting ACHE activity. It can be considered as economic therapeutic option against cognitive disorders associated with decline in cholinergic neurotransmission. This new finding will

beneficially help develop innovative technologies, design new types of functional foods, and promote both El Hammoum production and relevant food processing industry in the future. Finally, further research is needed toward isolation and identification of active principles present in the different extracts which could possibly be exploited for pharmaceutical use.

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