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Effect of heat treatment on polyphenolic compounds and antioxidant activity of lentils (*Lens culinaris*)

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Abstract: *The current research involves the study of the effect of thermal treatment in boiling water (cooking) on the nutrient components and phytochemical contents of lentils. The changes of phenolic compounds and antioxidant activity of lentils before and after cooking; as well as the recovered water are investigated. Total polyphenol, flavonoid and tannin content were determined spectrophotometrically using standards methods and LC-MS/MS. Antioxidant activity of methanolic extracts was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethyl benzo thiazoline-6-sulfonic acid)) assays. As compared to the raw lentils, cooking caused significant ($p < 0.05$) decreases in total phenolic content (TPC), total flavonoid content (TFC), DPPH and ABTS free radicals scavenging activity. The obtained results indicated that the polyphenol content of cooking water was significantly higher than that of cooked and raw lentils; however the most important antioxidant activity was recorded for raw lentils. Several phenolic compounds were identified in fresh, cooked lentils and cooking water. The major compound for the three samples is tannic acid with a very remarkable value in the cooking water; while the minor compounds are naringenin for cooked lentils, apigenin for raw lentils and cooking water. Neoformed compounds have been also detected in the cooking water such as: quercetin, hesperitin, tr-caffeic acid and chlorogenous acid in the cooking water.*

Keywords: Lentils, cooking, polyphenol; antioxidant activity, LCMS/MS

Introduction

Pulses are the edible seeds of plants in the legume family, including chickpeas, lentils, dry peas and beans. They are produced and consumed widely around the world [1]. They are the essential food resources which contribute to the nutritional wellbeing of various human diets [2]. Leguminous seeds belong to plant foods which are generally rich in phenolic compounds, including condensed tannins [3; 4]. These compounds possess various biological properties that provide a number of benefits, including antioxidant, apoptotic, anti-aging, anti-carcinogenic and anti-inflammatory activities, cardiovascular protection and enhancement of endothelial function. Moreover, polyphenols could also inhibit angiogenesis and cell proliferation activity [5]. The antioxidant capacity [3] and the antimutagenic [6], apoptosis related [7], and antiproliferative effects of legumes are highly associated with the presence of phenolic compounds [8].

The quantity and quality of polyphenols in vegetables might vary greatly depending on various factors. Another set of factors originates when the plant leaves the farm where it was grown and is transported to the consumer. Factors related to the consumer food preservation and preparation also affect the rate of these substances, sometimes with significant impact [9]. Cooking causes some physicochemical changes in pulses, including gelatinization of starch, denaturation of proteins, solubilization of some of the polysaccharides, and softening and breakdown of the middle lamella, a cementing material found in the cotyledon [10]. Prior to cooking, soaking is the preliminary step that helps to increase the tissue texture, shorten the cooking time, and further reduce and further reduce the hardness of the seed. High pressure processing technology can maintain nutritional value and quality of food products (flavour, colour, biological active components) [11]. Several pulses, including lentils (*Lens culinaris*) are not consumed raw; they should be cooked before consumption. The effect of cooking on antioxidant capacity and phenolic compounds of these foods has not been extensively studied [12]. In this framework, the main objective of the present work was to investigate the effect of cooking in boiling water on the phenolic compounds and antioxidant activity of lentils (*Lens culinaris*). To reach this goal, total phenolic, flavonoid and tannin contents are evaluated on the raw and cooked lentils, as well as the recovered cooking water. The phenolic compounds of studied lentils were also tentatively identified and quantified by mass; as well and an in vitro evaluation of the antioxidant activity was also carried out.

Materials and methods

Raw materials

The research was performed on lentils (*Lens culinaris* L.) of Metropole variety. It is the most marketed and consumed variety in Algeria. Lentils were provided by National Centre for Control and Certification of Seeds and Plants (C.N.C.C, Constantine, Alegria).

Samples preparation

From raw lentils

Lentils were manually sorted and cleaned, to remove all impurities (broken grains, infested grains, foreign grains, etc.), and ground into fine powder ($< 250 \mu\text{m}$) using an electric grinder (Group SEB, French). The obtained powder was stored in dark and airtight containers until being used.

From cooked lentils

Approximately 100 g of lentils were immersed in a stainless steel vessel with a lid containing 600 mL of water and cooked at 100°C for 45 min. The samples were drained and kept at -20°C . After 24 hours, the frozen lentils were milled and freeze-dried.

From cooking water

The water used for cooking was recovered and stored at -20°C . After 24 hours, the formed water crystals were freeze-dried.

Preparation of phenolic extracts

The extraction of phenols was carried out according to the procedure described by Mujica et al. [13]. To obtain the extract, 1 g of powder was dissolved in 25 acidified methanol with 0.1% HCl (2N). The mixture was subjected to sonication treatment (Jeiotech, USA), and then centrifuged at 1800 g for 15 min. The residue was re-extracted with 25 mL of methanol and centrifuged at 1800 g for 15 min. The methanolic phase was recovered, filtered through $0.45 \mu\text{m}$ pores filters, and finally evaporated under vacuum at 45°C to remove the solvent. The extracts were used both for the determination of total phenols and flavonoids.

For LC-MS/MS analysis, the extraction procedure was carried out as described by Ertas et al. [14]. In brief, 100 g of each powder were mixed with 300 mL of methanol and subjected to maceration for 24 h at room temperature. To obtain the crude extracts, the solvent was removed under vacuum at 30°C using a rotary evaporator. Afterwards, the dry residue was reconstituted in methanol at a concentration of 1000 mg/L, and then filtered through microfibre filters having pores of $0.45 \mu\text{m}$.

Quantitative determination of total bioactive compounds

Total phenolic content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method using a calibration curve of pyrocatechol ($R^2 = 0.9969$) [14, 15]. Briefly, in a 96 well plate, 4 μL of each extract were mixed with 4 μL of Folin-Ciocalteu reagent and 12 μL of sodium carbonate solution (2% w/v). The plate was incubated for 120 min in the dark at room temperature. The absorbance was measured at 760 nm using Microplate Reader with 96 well plates (BioTek Instruments, Eon, USA). Pyrocatechol was used as a reference standard, and the total phenolic content was expressed as μg of pyrocatechol equivalents (PE) per mg of dry matter ($\mu\text{g PE/mg dm}$).

Total flavonoid content

The total flavonoid content (TFC) was determined according to Moreno et al. [16]. In a 96 well plate, 20 μL of sample extracts were mixed with 172 μL of ethanol (80%) and 4 μL of a 10% solution of $\text{Al}(\text{NO}_3)_3$. After incubation in the dark at room temperature for 40 min, the absorbance of the reaction mixture was read at 415 nm. The results were expressed as μg of quercetin equivalents (QE) per mg of dry matter (μg QE/mg dm) based on a quercetin standard curve made in the same conditions ($R^2 = 0.9986$).

Total tannin content

The total tannin content (TTC) was determined as reported in Sadasivum and Manickam [17]. Each powder previously prepared (0.5 g) was mixed with 75 mL of distilled water. The mixture was boiled for 30 min, and then centrifuged at 2000 rpm for 20 minutes. The supernatant was recovered and the final volume was adjusted to 100 mL.

One mL of each extract was added to 75 mL of distilled water, 5 mL of Folin-Denis reagent and 10 mL of sodium carbonate solution (0.1 N). The absorbance was then measured at 700 nm after 30 min of incubation in the dark. The total tannin content was expressed as μg of tannic acid equivalents per mg of dry matter (μg TAE/mg dm) by means of a tannic acid standard curve made in the same conditions ($R^2 = 0.962$).

LC-MS/MS determination of the phenolic compounds

The LC-MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The UHPLC system was composed by LC-30AD binary pumps, a DGU20A3R degasser, a CTO-10ASvp column oven, and a SIL-30AC auto sampler. The chromatographic separation of phenols was achieved using a reverse-phase C18 Inertsil ODS-4 analytical column (150 mm $\times$ 4.6 mm, 3 μm). Mobile phase consisted of solvent A (0.1%, v/v, solution of formic acid in water) and solvent B (0.1%, v/v, solution of formic acid in methanol) at different ratios. The gradient program with the following proportions of solvent B was applied; time (min), B %: (0, 35), (5, 50), (10, 90), (12, 90), (13, 35). The flow rate was 0.5 mL/min, the column temperature was fixed at 40°C and the injection volume was 2 mL.

MS detection was performed using a Shimadzu LCMS 8040 triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. The optimum ESI conditions were determined to be: an interface temperature 350°C, desolvation line temperature 250°C, heat block temperature 400°C, nebulizer gas flow (N_2) 3 L/min, and drying gas flow (N_2) 15 L/min. The LC-MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan).

Evaluation of the total antioxidant capacity

DPPH assay: The antioxidant activity was evaluated on the basis of the scavenging activity of the DPPH (1,1-diphenyl-2-picrylhydrazyl) as described in Ertas et al. [14]. Briefly, 40 μL of each extract at

different concentrations (10 to 100 ppm) were added to 160 μ L of DPPH solution (0.1 mM in methanol). After 30 min in the dark, the decrease of absorbance was measured at 517 nm by spectrophotometry (Bioteck, Eon, USA) against a blank (methanol with DPPH).

ABTS assay: Extracts were analyzed also for their capacity to scavenge the ABTS radical (2,2'-azinobis (3-ethyl benzo thiazoline-6-sulphonic acid) diammonium salt) accordingly to the method proposed by Ertas et al. [14]. In a 96 well plate, 20 μ L of each extract (0 to 1000 ppm) were added to 1.60 mL of ABTS reagent previously prepared. After 30 min in the dark, the decrease of absorbance was read at 734 nm.

Extract concentration providing 50% inhibition (IC50%) was calculated from the graph by plotting inhibition against extract concentration. Butylated hydroxyanisole (BHA) and α -Tocopherol (α -TOC) were used as reference.

Statistical analysis

All analyses were carried-out in triplicate and the experimental data were reported as means $\pm$ standard deviation. Analysis of variance (ANOVA) was performed on the experimental data using XLSTAT software. The differences were considered statistically significant at $p < 0.05$.

Results and Discussion

The effect of cooking on total phenolic content

The results of the evaluation of total phenolic contents (TPC) in raw, cooked lentils and cooking water are given in Table 1, as pyrocatechol equivalents by reference to standard curve. Phenolic contents showed significant differences among samples ($p \leq 0.05$). Lentils showed the significantly higher ($p \leq 0.05$) total phenolic content in their raw samples (172.36 μ g PE/mg dm) compared with their cooked samples (72.36 $\pm$ 2.78 μ g PE/mg dm). Cooking treatment caused significant losses (41.98%) of total phenolic compounds. This indicates the breaking down of polyphenols in these vegetables types during cooking. The cooking water showed the highest level of TPC (244.12 μ g PE/mg dm) compared with raw and cooked lentils, an increase of 141.69%.

This increase in TPC in cooking water is in fact due to the solubilization and the diffusion of phenolic compounds following the bursting of cell structures by the heat. Thermal treatment causes many changes in the physical characteristics and phytochemicals content, in particular, the antioxidants found in vegetables [18, 19, 20]. Dewanto et al. [21] reported that thermal degradation caused by cooking is compensated by facilitated extraction of polyphenols from vegetable tissues. These compounds are therefore detected in higher amounts after cooking. According to Makris and Rossiter [22] and Renard [23], cooking in boiling water is the least effective in increasing phenolic compounds, due to the solubilization of polyphenols in cooking

water during boiling treatment. Similarly, Kutos et al. [24] have also shown that cooking produces significant levels of reduction in phenolic compounds (from 52% to 86% for pulses after 30 to 120 minutes of cooking). Other authors suggest that the apparent decrease in polyphenols during cooking is due to a leaching or diffusion [22].

The effect of cooking on total flavonoid content

Total flavonoid content (TFC) of the three different samples is shown in Table 1. Flavonoid content also showed significant differences among samples ($p \leq 0.05$). As in the polyphenols, total flavonoid content decreased significantly ($p \leq 0.05$) compared with raw lentils after cooking. The percentage decrease of flavonoid content in cooked lentils was 41.19% when compared with fresh lentils. This may be related to a disruption of cell walls and liberation of flavonoids, which caused their diffusion into the cooking water, resulting in an increase of about 428.37% compared to the raw state. According to Charles and Benbrook [9], the flavonoid content varies significantly depending on the variety and the degree of maturity. Indeed, referring to literature, Jones and Vogt [25], flavonoids may exist in the *form of heterosides*, in which one or more hydroxyl groups are combined with sugars. The presence of this osidic fraction makes flavonoids very soluble in water which explains the obtained results.

The effect of cooking on total tannin content

Total tannin contents (TTC) of raw, cooked lentils and cooking water are significantly different ($p \leq 0.05$). As illustrated in Table 1, lentils showed the significantly higher TTC in their cooked samples compared with their raw samples. However, the cooking water showed the highest level of TTC ($9.06 \pm 0.03 \mu\text{g TAE} / \text{mg dm}$) compared with raw and cooked lentils.

According to Vidal-Valverde et al. [26], lentils are rich in tannins. Among these compounds catechin, polymer of flavanols and procyanidins [27], is less soluble [28]. Similarly, flavonoids such as flavonol glycosides and flavones have been identified in the seed coat [29, 30], although this represents only a small percentage of the whole grain weight (from 8% to 11%) [31]. Lea and Arnold [32] reported that the polymerization degree of the parietal tannins (catechin and procyanidin) is greater than that of the intracellular tannins. Amrani et Glories [33] and Macheix et al. [34] suggest that tannins are associated with proteins or polysaccharides in vacuolar membranes and cell walls, which would limit their extractability and reactivity. Moreover, condensed tannins cannot be thermally degraded, which explains the low tannin content in the raw lentils.

In the case of hydrolyzable tannins, cooking in boiling water allows the extraction of tannins bound to sugars (sugar polyester) [35]. The sugar is often a glucose or an acid (quinic and gallic), which explains the high tannin content in the cooked lentils.

Bruneton [36] reported that some phenolic acids, produced by thermal degradation of hydrolysable tannins, tend to polymerize in solvents. They can react with each other by *complexation* to reform certain tannic forms, such as gallotanins (glucose and gallic acid ester) and ellagitannins (glucose and ellagic acid ester), cyanarines (caffeic acid ester) and tannic acid, tannic acid, which could explain the high tannin content in the cooking water.

Table 1. Total phenolic content, total flavonoid content and total tannin content of the three different samples

Parameter	Raw lentils	Cooked lentils	Cooking water
TPC ($\mu\text{g PE mg}^{-1}$)	172.36 ± 2.78^c	72.36 ± 2.78^b	244.12 ± 4.46^a
TFC ($\mu\text{g QE mg}^{-1}$)	42.82 ± 0.53^c	17.64 ± 0.10^b	183.43 ± 3.29^a
TTC ($\mu\text{g TAE mg}^{-1}$)	0.02 ± 0.01^c	4.80 ± 0.15^b	9.06 ± 0.03^a

TPC, total phenolic content; TFC, total flavonoid content; TTC, total tannin content.
 PE, pyrocatechol equivalents; QE, quercetin equivalents; TAE, tannic acid equivalents.
 Different letters in row indicate significant difference at $p \leq 0.05$.

Identification and quantification of individual polyphenols by LC-MS/MS

Comparing the retention time (Figure 1. a) of standards with those recorded in the different chromatograms (Figure 1. b, c and d) allowed the identification of the phenolic compounds shown in Table 2.

As it can be observed from the results, several phenolic compounds were identified in fresh, cooked lentils and cooking water. It was found that the major compound for the three samples is tannic acid with a very remarkable value in the cooking water. It is suggest that tannic acid, which is polyester of glucose and gallic acid (hydrolyzable tannin) soluble in water, easily diffuses into cooking water after thermal degradation of the cell wall and membrane on the one hand, and that the polymerization of glucose and gallic acid lead to its neoformation, which can justify the tannin determination result on the other hand. Moreover, other phenolic compounds have been neoformed in the cooking water such as: tr-caffeic acid, hesperetin, chlorogenic acid, malic acid and quercetin. In fact, the neoformation of these compounds can be explained by the thermal degradation of quercetin glycosides which leads to the cleavage between sugar and the aglycone part and thus increases the amount of quercetin measured; thermal degradation of hesperidine (*hesperetin 7-rutinoside*) [37]; chlorogenic acid is an ester of caffeic acid and (L)-quinic acid [38]; and caffeic acid which is synthesized by hydroxylation of paracoumaric acid (in ester form).

The levels of other phenolic compounds are very important in cooking water than in raw and cooked lentils, this explains the result of the determination of total polyphenols, flavonoids and tannins. Therefore,

total polyphenols will be able to be exposed to reactions, the main one being oxidation, to diffuse more freely and interact with the present macromolecules, mainly the parietal polysaccharides [39].

Two compounds, rutin and hesperidin, are present in raw lentils and absent in cooked lentils and cooking water, this is probably explained by the structural modification of the phenolic compounds after cooking and their thermal degradation. Minority compounds are naringenin for cooked lentils, apigenin for raw lentils and cooking water.

Table 2. Identification and quantification ($\mu\text{g}/\text{mg}$) of single phenols in raw, cooked lentils and cooking water by LC-PDA-MS

No.	Compounds	RT ^a	Parent ion (m/z) ^b	R ^{2c}	RSD% ^d	LOD/LO Q ($\mu\text{g}/\text{L}$) ^e	Raw lentils	Cooked lentils	Cooking water
1	Quinic acid	3.32	190.95	0.9927	0.0388	22.3/74.5	949.50	529.95	5830.67
2	Malic acid	3.54	133.05	0.9975	0.1214	19.2/64.1	-	-	2168.93
3	tr-Aconitic acid	4.13	172.85	0.9933	0.3908	15.6/51.9	-	-	-
4	Gallic acid	4.29	169.05	0.9901	0.4734	4.8 / 15.9	125.95	133.71	702.65
5	Chlorogenic acid	5.43	353	0.9932	0.1882	7.3 / 24.3	-	-	14.15
6	Protocatechuic acid	5.63	152.95	0.9991	0.5958	25.8/85.9	67.07	65.49	533.23
7	Tannic acid	6.46	182.95	0.9955	0.9075	10.2/34.2	3734.71	4127.67	30597.25
8	tr- caffeic acid	7.37	178.95	0.9942	1.0080	4.4 / 14.7	-	-	18.64
9	Vanillin	8.77	151.05	0.9995	0.4094	10.1/33.7	-	-	-
10	p-Coumaric acid	9.53	162.95	0.9909	1.1358	15.2/50.8	17.81	-	556.26
11	Rosmarinic acid	9.57	358.9	0.9992	0.5220	10.4/34.8	-	-	-
12	Rutin	10.18	609.1	0.9971	0.8146	17.0/56.6	13.94	-	-
13	Hesperidin	9.69	611.1	0.9973	0.1363	21.6/71.9	17.71	-	-
14	Hyperoside	10.43	463.1	0.9549	0.2135	12.4/41.4	185.03	108.18	578.02
15	4-OH Benzoic acid	11.72	136.95	0.9925	1.4013	3.0/10.0	88.35	68.55	345.37
16	Salicylic acid	11.72	136.95	0.9904	0.6619	4/13.3	77.92	61.01	287.31
17	Myricetin	11.94	317	0.9991	2.8247	9.9/32.9	-	-	-

18	Fisetin	12.61	284.95	0.9988	2.4262	10.7/35.6	297.76	157.26	259.90
19	Coumarin	12.52	146.95	0.9924	0.4203	9.1 / 30.4	-	-	-
20	Quercetin	14.48	300.9	0.9995	4.3149	2.0 / 6.8	-	-	178.69
21	Naringenin	14.66	270.95	0.9956	2.0200	2.6 / 8.8	5.47	2.15	12.71
22	Hesperetin	15.29	300.95	0.9961	1.0164	3.3/ 11.0	-	-	9.70
23	Luteolin	15.43	284.95	0.9992	3.9487	5.8 / 19.4	132.54	71.84	587.11
24	Kaempferol	15.43	284.95	0.9917	0.5885	2.0 / 6.6	133.28	76.46	578.00
25	Apigenin	17.31	268.95	0.9954	0.6782	0.1 / 0.3	3.16	-	9.15
26	Rhamnetin	18.94	314.95	0.9994	2.5678	0.2 / 0.7	-	-	-
27	Chrysin	21.18	253	0.9965	1.5530	0.05/ 0.17	-	-	-

^aRT: Retention time; ^bParent ion (m/z): Molecular ions of the standard compounds (mass to charge ratio); ^cR²: coefficient of determination; ^dRSD: relative standard deviation; ^eLOD/LOQ ($\mu\text{g/L}$): Limit of detection/Limit of quantification; - not detected

Figure 1. LC-MS/MS chromatograms of the methanolic extract of a: standard mix, b: raw lentils, c: cooked lentils and d: cooking water. Peaks numbers correspond to those reported in Table 2

The effect of cooking on antioxidant activity

Two different antioxidant activity assays were used to evaluate the effect of cooking on the antioxidant capacity of studied lentils. Table 3 displays the total antioxidant capacity of raw, cooked lentils and cooking water. DPPH radical scavenging activity expressed in IC_{50} values of the different methanolic extracts ranged from $14.46 \mu\text{g ml}^{-1}$ to $35.14 \mu\text{g ml}^{-1}$. In the ABTS assay, IC_{50} values ranged from $100.57 \mu\text{g ml}^{-1}$ to $147.84 \mu\text{g ml}^{-1}$. The IC_{50} is generally inversely proportional to the antioxidant capacity of the phenolic compounds. According to the obtained results, antioxidant activity appears to be affected by cooking for both tests (DPPH and ABTS). All studied extracts showed scavenging activity of DPPH and ABTS radicals. The highest activity is recorded in the raw lentil extract, followed by the cooked lentil extract. However, the significantly lower ($p < 0.05$) antioxidant capacity was observed in cooking water when compared with the antioxidant capacities of fresh and cooked lentils.

Table 3. IC_{50} ($\mu\text{g ml}^{-1}$) values of methanolic extracts for free radical scavenging activity by DPPH and ABTS radicals

Parameter	DPPH assay	ABTS assay
α -TOC	16.30 ± 0.79	9.88 ± 0.23
BHT	58.86 ± 0.50	1229 ± 0.67
Raw lentils	14.46 ± 0.23^c	100.57 ± 1.34^c
Cooked lentils	30.35 ± 0.76^b	110.79 ± 1.23^b
Cooking water	35.14 ± 0.90^a	147.84 ± 0.65^a

Different letters indicate significant difference at $p \leq 0.05$

The obtained results in this study do not agree with those reported by Xu and Change [40]. Rice-Evans et al. [41] report that the antioxidant activity of phenolic compounds in vegetables depends on the structure, in particular the degree and positions of the hydroxyl groups. Likewise, Makris and Rossiter [22] suggest that the cooking caused a number of changes in amount and structure of polyphenols, which could affect the antioxidant capacity. This may explain the decrease in antioxidant activity despite increasing levels of total polyphenols, flavonoids and tannins. Furthermore, each phenolic compound has a degree of affinity (high or low) for the free radical DPPH, which could influence the observed variations in antioxidant potency [42]. Indeed, different compounds other than polyphenols, resulting from the Maillard reaction, could also be responsible for the antioxidant capacity [43]. Nicoli et al. (1999) [44] was indicated that the decrease in

antioxidant capacity was relative to the loss of native antioxidant molecules, as well as the production of new prooxidant compounds.

Conclusion

The findings from the present study indicate that the heat treatment affect significantly total polyphenols, flavonoids and tannins as well as antioxidant capacity of lentils. The highest content of polyphenols was recorded for cooking water with an increase of 141.69% and the lowest for lentils cooked with a reduction of 41.98%. The same finding was observed for the flavonoid content, where the cooking caused a reduction of 41.19%. However, cooking treatment showed a positive effect on the tannin content of cooked lentils compared to that of raw lentils. The tannins evaluated in the cooking water are markedly high. Neoformed compounds were detected by LCMS/MS. As for the antioxidant activity (DPPH and ABTS), the cooking process have shown a negative impact. The highest activity was found in the raw lentil extract and the lowest was found in the cooking water.

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