Malting Process Effects on Antioxidant Activity of Finger Millet and Amaranth Grains

Sara Najdi Hejazi[1] & Valérie Orsat[1]

[1] Bioresource Engineering Department, McGill University, Ste-Anne-de-Bellevue, Canada

Correspondence: Sara Najdi Hejazi, Bioresource Engineering Department, McGill University, Qc, H9X 3V9, Canada. Tel: 1-514-992-4192. E-mail: sara.najdihejazi@mail.mcgill.ca

Abstract

Finger millet (Eleusine coracana) and amaranth (Amaranthus caudatus) are two nutritious and gluten-free grains with high contents of phenolic compounds. Phenols are known as the main source of antioxidants, with numerous health benefits. Being rich in phenol makes these grains good choices for the functional food industry. In this study, the effects of malting/germination factors, duration and temperature, on the phenolic content and antioxidant activities of these grains are thoroughly investigated and optimized. Based on a central composite design, the grains were germinated for 24, 36, and 48 hrs at 22, 26, and 30 °C. Both temperature and duration factors are found to be significantly influential on the monitored quantities. While malting of amaranth grains for 48 hrs at 26 °C increased the total phenol content four times, in case of millet, a 25% reduction was observed. Linear correlations between the included phenol content and antioxidant activity in terms of DPPH and ABTS scavenging activities were observed.

Keywords: Amaranth, finger millet, malting/germination process, phenol content, ABTS, DPPH, colorimetric properties

1. Introduction

Even though oxidation reactions are important for cellular metabolism, they may threaten cell normal functions, especially in the case of chain reactions (Banerjee, Sanjay, Chethan, & Malleshi, 2012). When a chain oxidation reaction initiates, excessive amounts of free radicals are produced that may cause serious damage or death to the cell. These excessive free radicals may cause oxidative stress (Hegde, Rajasekaran, & Chandra, 2005). Oxidative stress plays a significant role in many human body malfunctions, most importantly coronary heart disease and cancer (Najdi Hejazi, 2012; Saleh, Zhang, Chen, & Shen, 2013).

Antioxidants are molecules that prevent oxidation of other components by being oxidized themselves (Viswanath, Urooj, & Malleshi, 2009). In fact, antioxidants are known as reducing agents that terminate the chain reactions through removing free radical intermediates and inhibit excessive oxidation reactions. In the food industry, antioxidants act as natural preservatives in the products by preventing degradation of their lipid and protein contents (Venskutonis & Kraujalis, 2013). In cereal grains, polyphenolic compounds are assumed to be the main source of antioxidants (Towo, Svanberg, & Ndossi, 2003). Naturally, polyphenols are responsible in the defense of plants against ultraviolet radiations and pathogens aggression (Banerjee et al., 2012). These compounds are referred to as the secondary metabolites of plants. Epidemiological studies have proven that long term consumption of cereal grains, which contain appreciable amounts of phytochemicals, may significantly reduce oxidative stress in the body and keep the desired balance between oxidants and antioxidants levels (Dykes & Rooney, 2007; Hegde et al., 2005).

Recently, with increasing concerns about worldwide food security, increasing attention is given on the cultivation of drought-resistant crops that can be grown in arid and semi-arid regions (Malleshi & Desikachar, 1986). Among them, millet and amaranth grains are gaining significant appreciation. Having good sensory qualities, short growing season, and appropriate nutrient profiles besides being gluten-free are the main factors for this attention (Belton & Taylor, 2002; Charalampopoulos, Wang, Pandiella, & Webb, 2002).The high phenolic contents of these seeds could represent a good source of antioxidant, particularly in the arid regions, where other commercial crops cannot be grown (de la Rosa et al., 2009; Shobana et al., 2012). Among a vast variety of amaranth grains, three of them are of more importance, Amaranthus caudatusm, Amaranthus cruentus,

and Amaranthus hypochondriacus (Venskutonis & Kraujalis, 2013). Millets are categorized into two groups; major millets, which include species that are most widely cultivated, and minor millets (Issoufou, Mahamadou, & Guo-Wei, 2013). While Sorghum and Pearl Millet belongs to the first category, Finger Millet, Foxtail Millet, Little Millet, Barnyard Millet, Proso Millet, and Kodo Millet are mostly recognized as minor millets (Obilana & Manyasa, 2002). In this study, total phenolic content and antioxidant activities of Finger millet (Eleusine coracana) and Amaranth (Caudatus amaranthus) are investigated. In addition, the effect of a malting/germination process on the resulting phenolic content and antioxidant activities is explored.

A study performed on two varieties of Caudatus amaranth, Centenario and Oscar Blanco, demonstrated that their total phenolic contents were 98.7 and 112.9 mg/100 g, respectively, using Gallic acid as the reference (Repo-Carrasco-Valencia, Pena, Kallio, & Salminen, 2009). In addition, the authors reported that antioxidant activities using DPPH method were 410 and 398.1 μMol Trolox/g for Centenario and Oscar Blanco, respectively. The ABTS activities were reported at 827.6 and 670.1 μMol Trolox/g, respectively.

Pasko et al., (2009) studied the phenolic contents and antioxidant activity of two varieties, Aztec and Rawa, of Cruentus amaranth. It has been shown that for the native seeds, average total phenols were 2.95 and 3.0 mg GAE/kg, average DPPH activities were 4.42 and 3.15 mMol Trolox/kg, and average ABTS activities were 12.71 and 11.42 mMol Trolox/kg for Aztec and Rawa seeds, respectively. Besides, in the performed study, effects of germination duration on these quantities were investigated. Grains were germinated at room temperature between four to seven days in daylight or darkness. It has been found that sprout antioxidant activities depend on the growth duration, where maximum values were observed at the 4th day. A significant increase in the DPPH and ABTS scavenging activities and a slight decrease in the total phenolic content were reported. Besides, effects of light during germination on the antioxidant activities were claimed to be significant. For Rawa seed, ABTS was in the range of 112.9 to 151.3 mMol Trolox/kg for the grains that were germinated in daylight and 78.8 to 176.1 for those germinated in darkness. ABTS values for Aztec were in the range of 133.1 to 222.1 and 99.5 to 17.5 mMol Trolox/kg for sprouts grown in daylight and darkness, respectively. Finally, significant linear correlations between ABTS and DPPH radical scavenging activities (R^2=0.87), as well as between total phenol content and ABTS and DPPH (R^2=0.98) were obtained (Paśko et al., 2009). Nevertheless, the present authors believe that this conclusion may not remain accurate if the values of native grains were included. This is due to the fact that the phenol content did not significantly change after germination, while the antioxidant activities remarkably (more than 10 times) increased throughout the germination process.

In another recent study, effect of germination on the phenolic content and DPPH radical scavenging activity was investigated for Cruentus amaranth (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010). Grains were germinated for 98 hrs at 10 ℃. Total phenol content increased from 21.2 up to 82.2 mg GAE/100 g after germination. A slight decrease in the DPPH activities from 28.4 to 27.1 mg Trolox/100 g was observed.

For finger millet, Siwela et al.(2007) reported a low phenolic content for white varieties with values below 0.09 mg GAE/100 mg. Comparably higher amounts were obtained for brown finger millets, ranging from 0.34 to 1.84 mg GAE/100 mg. The results are aligned with findings of Ramachandra et al. (1977), who detected phenolic content of 0.06 to 0.1 mg/100 mg, in Chlorogenic acid equivalent (CGA), for white and 0.34 to 2.44 mg/100 mg in brown varieties. A similar seed color dependency was observed by Chethan and Malleshi (2007), who reported pholyphenol content of finger millet ranging from 0.3 to 0.5 % (GAE) in white, versus 1.2 to 2.3 % in brown seeds. Antioxidant activity of white finger millets were reported 37.5 to 75.9 mM Trolox/Kg, while these values for the dark varieties increased up to 117.1 to 195.4 (Siwela et al., 2007). DPPH activity for finger millet was observed to be 1.73 mg Trolox/g by Sreeramulu et al. (2009). They reported 373.15± 70.07 mg GAE/100 g for the finger millet phenolic content (Sreeramulu et al., 2009).

Towo et al.(2003) investigated the effect of germination process on the total phenolic content of finger millet. Grains were germinated in darkness at 25 ℃. Germination duration was not specified in their paper. Total phenol decreased from 4.2 to 3.3 mg/g in Catechin Equivalents (CE). Sripriya et al. (1996) reported a similar decrease in phenolic content during germination of finger millet. In their study, total phenol decreased from 102 to 67 mg CGA/100 g (Sripriya et al., 1996). Again, germination parameters including duration were not specified.

The observed diversity in the phenolic contents and antioxidant properties of amaranth and millet grains in the literature is due to differences in employed extraction approaches and sample preparation. Besides, having different evaluation procedures as well as different reference calibrated curves in expressing the results are other main factors for these discrepancies.

It has been shown that food processing steps may alter the phenolic contents of cereal and pseudo-cereal grains (Kunyanga, Imungi, Okoth, Biesalski, & Vadivel, 2012; Queiroz, Manólio, Capriles, Torres, & Areas, 2009;

Saleh et al., 2013; Shobana et al., 2012). One of the simplest and widely employed pre-treatments is the malting/germination process, where grains are soaked, germinated, dried, and ground to a flour (Najdi Hejazi, Orsat, Azadi, & Kubow, 2015; Swami, Thakor, & Gurav, 2013; Traoré, Mouquet, Icard-Vernière, Traore, & Trèche, 2004). In the present study, effects of malting/germination parameters, duration and temperature, on the total phenol content and antioxidant activities of finger millet and amaranth grains were investigated and optimized.

2. Material and Methods

2.1 Materials

Brown finger millet (Eleusine coracana) was procured from University of Agricultural Sciences (Dharwad, India) and amaranth (Amaranthus caudatus L. (love-lies-bleeding)) seeds were purchased from a local market (Bulk Barn store, Qc., Canada).

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu reagent, chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and potassium persulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol was obtained from Commercial Alcohols (Industrial and Beverage Alcohol Division of Green-field Ethanol Inc., Ontario, Canada). Double distilled water (ddH2O) was prepared using Simplicity TM water purification system (Millipore, USA). All other reagents were of analytical and HPLC grades (Teow et al., 2007).

2.2 Malting/Germination Process

Finger millet and amaranth seeds were cleaned thoroughly with sterile water and surface-air dried by airflow. The seeds were steeped (seed/water ratio of 1:5 (w/v)) overnight, and sprouted at 22, 26, and 30 °C in a B.O.D incubator (Benchmark Incu-Shaker Mini) for 24, 36, and 48 hrs. Germinated seeds were freeze-dried (FreeZone® 2.5 l Freeze Dry System, Labonco Corporation, MO, USA) for a week to reach constant dry weight. Native and germinated seeds were ground in an electric grinder (Bodum 10903, PRC, Intertek, USA) for further assays. Samples were stored in hermetic plastic containers at 4 °C.

2.3 Statistical Analysis

The experimental design used in the present study was a Central Composite Design (CCD) with two independent factors, germination duration and temperature, each at three levels, (24, 36, 48 hours) and (22, 26, 30 °C). All the experiments were performed in triplicate and the data presented as mean $\pm$ standard deviations. This design has 4 factorial, 4 axial, and 4 central points. For analyzing the data, JMP software version 11 (SAS Institute Inc., Cary, NC, USA) was used. The linear, quadratic, and combined effects of each factor were investigated using ANOVA analysis and regression models, and expressed as follows;

$$Y = \text{Intercept} + \beta_1 \times \text{Time} + \beta_2 \times \text{Temp} + \beta_{11} \times \text{Time}^2 + \beta_{22} \times \text{Temp}^2 + \beta_{12} \times \text{Time} \times \text{Temp}$$

In this equation, β_1 and β_2 represent the regression coefficients of the linear, β_{11} and β_{22} are the coefficients of the quadratic, while β_{12} indicates the interactive or bilinear effects. Time and Temp represent the deviation of independent germination variables, germination duration and temperature with respect to their centroids values, 36 hrs and 26 °C, respectively.

2.4 Dry Matter

Dry matters of flours were determined using AACC Method 44 – 15.02 (AACC, 1999).

2.5 Bioactive Compounds and Antioxidant Activity Determination

To determine the total phenol content and antioxidant activities, the phenolic compounds were initially extracted and their radical scavenging activities using ABTS and DPPH approaches were evaluated as follows;

2.5.1 Phenolic Compounds Extraction

Methanolic crude extracts were obtained from native and malted samples (Makkar, 2003). Briefly, 100 mg of fine flour sample was weighed in 1.5 ml eppendorf and 900 µL of methanol (90%) was added. The mixture was sonicated in a dark cold room for 30 min, and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was recovered and the pellet was re-centrifuged with addition of 600 µL methanol (90%) under the same conditions. Finally, the two supernatants were pooled and used for further total phenolic content and antioxidant scavenging activity assays.

2.5.2 Total Phenol Content

Total phenolic content was determined by adapting the Folin-Ciocalteu method (Singleton & Rossi, 1965). Initially, 2 ml ddH$_2$O was added to 100 µL of methanolic sample extract. Subsequently, 200 µL of Folin-Ciocalteu reagent (2N) was added to the mixture with vigorous vortexing. After 30 min of incubation in the dark at room temperature, 1 ml of aqueous sodium carbonate solution (7.5%) was added and vortexed. Absorbance of samples was read at 765nm (Ultraspec1000, Amersham Pharmacia Biotech, NJ, USA) against methanol as a blank after one hour more of incubation at ambient temperature. Using chlorogenic acid as the standard curve, total phenol was expressed in mg CGA/100g db.

2.5.3 DPPH Antioxidant Scavenging Activity

The approach to assess antioxidant activity using the DPPH free radical scavenging assay was adapted from the method of Martinez-Valverda et al. (2002).

To perform this assay, fresh DPPH stock solution (1mM) was made and appropriately diluted with absolute methanol to reach into an absorbance range of 0.5 to 0.9 unit. Briefly, 1.5 ml of the prepared DPPH solution was added to 100 µL of sample extracts, vortexed and incubated at room temperature for 30 min. The absorbance of the resulted solution was read at 517 nm against air as the blank. The free radical scavenging activity was estimated using standard curve of Trolox in different concentrations (0-500 mM) with R^2=0.992. Additional dilution was required if the absorbance was over the linear range of the standard. In this regard, for the finger millet samples, a dilution factor of 10 was required, while no additional dilution was needed for amaranth. The final results were expressed as mg Trolox equivalent per 100g on a dry basis (mg TE/100 g db).

2.5.4 ABTS Antioxidant Scavenging Activity

ABTS free radical scavenging assay was implemented after some modification of the method of Re et al. (1999). Initially, an ABTS (7mM) stock solution and a potassium persulfate (2.45 mM) solution were prepared. The working reagent was obtained by mixing the two stock solutions in equal quantities and left to be incubated in the dark for 12 hrs at room temperature. The procedure resulted into the production of radical cations of ABTS (ABTS$^+$). ABTS radical solution was diluted with 95% ethanol to obtain an absorbance of 0.7 $\pm$ 0.05 unit at 734 nm.

To assess the ABTS activities, 1.2 ml of the prepared working solution was added to 100 µL of the sample extracts. The absorbance of the mixture was determined within 1 to 3 min at 734 nm against air as a blank. For finger millet, a dilution factor of 10 was used to fit the absorbance in the standard curve. This dilution was not required for amaranth samples. The antioxidant activity was estimated using standard curve of Trolox in different concentrations (0-500 mM with R^2=0.99) and results were reported as mg TE/ 100g db.

2.5.5 Color Determination

Colorimetric properties of native and germinated samples were assessed using CIE L*a*b* (CIELAB) approach (Leon, Mery, Pedreschi, & Leon, 2006) by chromameter (Model- CR300, Konica-Minolta®, USA). The measurement was done by covering the 5 g of flour with the plastic food wrap with gentle spreading. The flour was fitted perfectly the diameter of the chromameter's testing window. The flour was located on the black material to prevent unwanted interference from ambient light and scattering of light from the source.

The values for L* (0 = black, 100 = white), a* (+ values = redness, - values = green), and b* (+ values = yellowness, -values=blueness) were obtained for each germination treatment. Total color difference ($\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$) is evaluated versus the control sample (native grain).

3. Results

The obtained results are presented for amaranth and finger millet in the following subsections.

3.1 Amaranth

Total phenol contents were assessed for the selected design combinations as described in section 0 and are presented in Table 1. From the table, it was observed that the germination process significantly increased the phenolic compounds in amaranth sprouts comparing to the initial value (71.55 $\pm$ 1.76 mg CGA100 g db) of native grains (GM0). An increase of nearly four times was observed for some of the germination treatments (e.g. GM5 cases). A similar increase was reported by Alvarez-Jubete et al. (2010), where the phenol content of amaranth sprouts showed a four-time increase (from initial 21.2 to final 82.2 mg GAE/100 g) after 96 hrs of germination at 10 °C. DPPH and ABTS scavenging activities for the studied cases are presented in Table 1 as well. Again, similar to the phenol content, an increasing trend in the antioxidant activities throughout germination was observed.

Table 1. Total phenol (mg CGA/100 g db) content and DPPH (mg TE/100 g db), and ABTS (mg TE/100 g db) activities of amaranth grain for the tested germination treatments

Exp. #	Germination factors and their levels		Total Phenol	DPPH	ABTS
	Temperature	Duration	(mg CGA/100 g db)	mg Trolox/100 g db	mg Trolox/100 g db
GM0	Control		71.55±1.76	43.16± 1.10	52.27± 2.13
GM1	22	24	126.80±3.59	56.71± 4.39	90.67± 1.79
GM2	22	36	187.54±14.00	59.84± 2.77	78.18± 3.99
GM3	22	48	220.56±7.47	75.21± 4.19	117.23± 8.51
GM4	26	24	144.01±1.47	70.11± 4.27	91.97± 3.74
GM5.1	26	36	262.52±9.53	108.02± 6.24	129.82± 4.51
GM5.2	26	36	264.68±0.75	108.62± 6.30	118.84± 7.06
GM5.3	26	36	259.82±16.00	109.00± 5.30	121.45± 8.21
GM5.4	26	36	261.75±7.17	110.67± 1.30	118.94± 5.60
GM6	26	48	250.01±5.89	91.89± 4.82	127.63± 8.92
GM7	30	24	118.82±0.67	60.39± 3.15	80.96± 1.28
GM8	30	36	160.84±1.97	69.18± 3.40	90.88± 3.15
GM9	30	48	269.15±0.25	107.74± 2.37	126.07± 9.22

The obtained phenol and antioxidant activities are graphically presented in Figure 1. From this figure, the following conclusions may be drawn. Firstly, germination process increased the bulk phenol content when comparing with the control sample (GM0). Secondly, the phenol content is a strong function of germination duration. Thirdly, having an increase and then decrease in the averaged phenol contents versus temperature confirms its second order dependency to the process temperature. Lastly, DPPH and ABTS activities are following a similar trend as the total phenol content indicating strong correlations between these two variables.
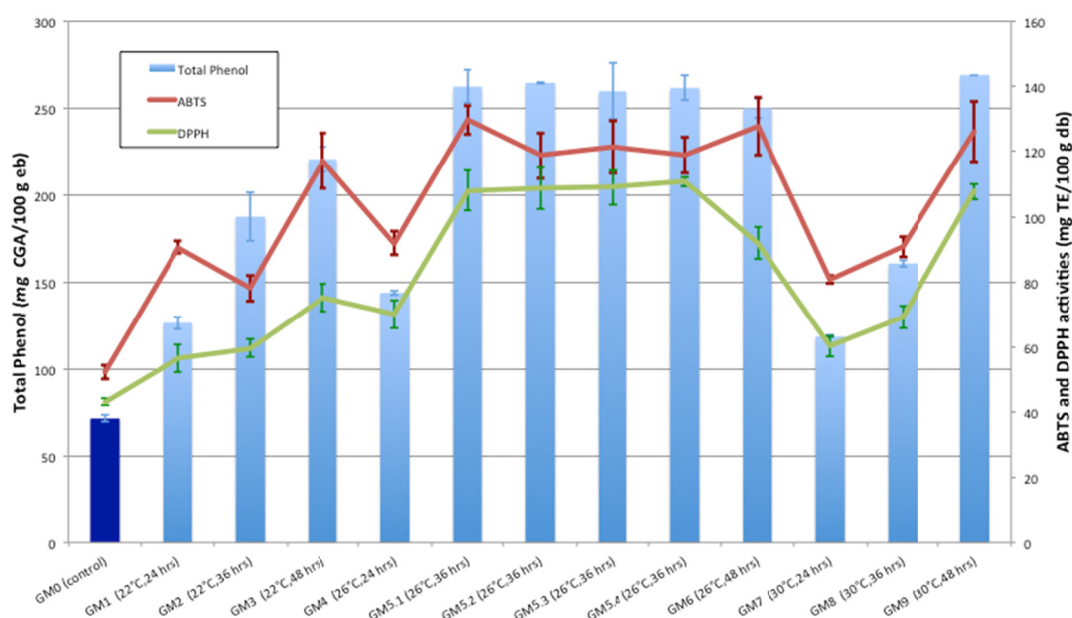

Figure 1. Total Phenol, ABTS, and DPPH activities of amaranth grain for the selected design factor combinations

In Table 2, reported data in the literature for the interested quantities, phenol content, ABTS, and DPPH antioxidant activities, are presented. For comparison, all the data are converted to be presented in mg/100g unit. Besides, data are categorized based on the different employed calibration curves; Gallic acid, Chlorogenic acid, and Trolox equivalents. Excluding a few exemption, our results are aligned with other reported data. Having different phenolic extraction approaches and different reading durations for ABTS and DPPH assays significantly influence the results, which is clearly observed in the reported data.

Table 2. Total phenol content, DPPH, and ABTS antioxidant activities (mg/100 g db) of amaranth grain

Amaranth Variety	Total Phenol (mg/100 g)	DPPH (mg/100 g)	ABTS (mg/100 g)
	Gallic acid equivalent	**Trolox equivalent**	**Trolox equivalent**
A. Caudatus Centenario (Repo-Carrasco-Valencia et al., 2009)	98.7	10261	20698
A. Caudatus Oscar Blanco (Repo-Carrasco-Valencia et al., 2009)	112.9	9961	16772
A. cruentus (raw) (Alvarez-Jubete et al., 2010)	21.2 ± 2.3	28.4 ± 1.3	-
A. cruentus (germinated)(Alvarez-Jubete et al., 2010)	82.2 ± 4.6	27.1 ± 2.7	-
A. Caudatus (Klimczak, Małecka, & Pachołek, 2002)	39.17	-	-
A. Paniculatus(Klimczak et al., 2002)	56.12	-	-
Amaranth (Mošovská, Mikulášová, Brindzová, Valík, & Mikušova, 2010)	104.1±2.2	-	-
A. cruentus var. Aztec (raw) (Paśko et al., 2009)	295 ±7	110.6 ± 12.5	302.85± 27.53
A. cruentus var. Aztec (sprout) (Paśko et al., 2009)	160 to 300	-	1972 to 4407
*A. cruentus*var. Rawa (raw) (Paśko et al., 2009)	300±42	78.8 ±7.5	285.83±30.03
*A. cruentus*var. Rawa (sprout) (Paśko et al., 2009)	150 to 250	-	2490 to 5560
*A. cruentus*var. Aztec seeds (PAŚKO et al., 2007)	-	110.6 ± 12.0	321.37± 23.03
*A. cruentus*var. Rawa seeds (PAŚKO et al., 2007)	-	78.8 ± 6	290.59 ± 16.27
Amaranth (Czerwiński et al., 2004)	14.72 to 14.91	-	-
Amaranth (Chlopicka et al., 2012)	271 ± 1	90.1 ± 8.5	-
Amaranth (Queiroz et al., 2009)	3170	-	-
		Gallic acid equivalent	
Amaranth (Asao & Watanabe, 2010)	51	22600	-
		Percentage basis	
A. hypochondriacus (López, Razzeto, Giménez, & Escudero, 2011)	57.1±1.0	86.93±1.4%	-
	Chlorogenic acid equivalent		
A. cruentus (Kunyanga et al., 2012)	1080	84.67±1.18%	-
		DPPH	
A. cruentus (Ogrodowska et al., 2012)	27.26 to 61.53	436.11 to 604.49	-
	Tannic acid equivalent		
A. cruentus (raw) (Gamel, Linssen, Mesallam, Damir, & Shekib, 2006)	516 to 524	-	-
A. cruentus (germinated) (Gamel et al., 2006)	368 to 420	-	-

Phenolic content data were analyzed using response surface methodology. Table 3 presents a summary of the obtained results for the performed calculations. The proposed response surface based on the selected design factors, $X_1 =$ (temperature – 26) and $X_2 =$ (duration – 36) is,

$$TP = 2.3183 \, X_1 + 58.3483 \, X_2 - 50.59 X_1^2 - 27.77 \, X_2^2 + 14.1425 \, X_1 \, X_2 + 249.7216 \tag{1}$$

Table 3. Summary of the ANOVA analysis of the responses for total phenol content and the corresponding parameter estimates of the different terms for amaranth grain

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	249.7216	12.592	19.83	**<.0001**
Temperature (X_1)	2.3183	11.2632	0.21	0.8437
Duration (X_2)	**58.3483**	11.26312	5.18	**0.0021**
Duration×Temperature ($X_1 X_2$)	14.1425	13.7945	1.03	0.3448
Temperature×Temperature (X_1^2)	**-50.59**	16.8948	-2.99	**0.0242**
Duration×Duration (X_2^2)	-27.77	16.8948	-1.64	0.1513

The analysis states that germination duration and square of germination temperature are significant terms with p<0.005 and p<0.05, respectively. A positive dependency of the obtained response surface to the duration indicates that the phenol content increases as germination time increases. Furthermore, having a positive coefficient for the quadratic term, X_1^2, means that a maximum should be expected in the constructed response surface.

The obtained response surface (Equation 1) is graphically plotted in Figure 2 (a). As it was pointed out, the surface has a maximum at 26.7 ℃ and 49.14 hrs. The predicted maximum value is 281.88 mg CGA/100 g db. This means that based on the conducted germination treatments and experiments, if it is desired to maximize the phenolic content and consequently its potential for higher antioxidant activity of selected amaranth grains, they should be germinated approximately for 48 hrs and at 26 ℃. This conclusion is based on the performed ANOVA study and constructed response surface. However, based on the obtained raw experimental data (Table 1), the best phenolic content was observed for seeds germinating for 36 hrs at 26 ℃. The relative difference is below 5% and may be neglected. The leverage plot (Figure 2 (b)) emphasizes on the appropriate prediction and interpolation of the experimental data by the proposed response surface. Since the confidence curves (dashed-line curves) crossed the horizontal line, the correlation of design factors is significant ($p<0.05$).
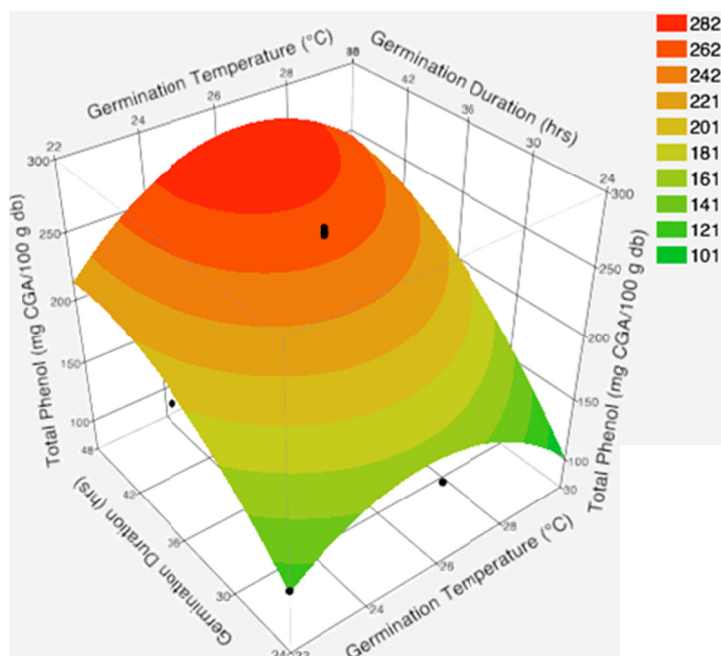
(a)
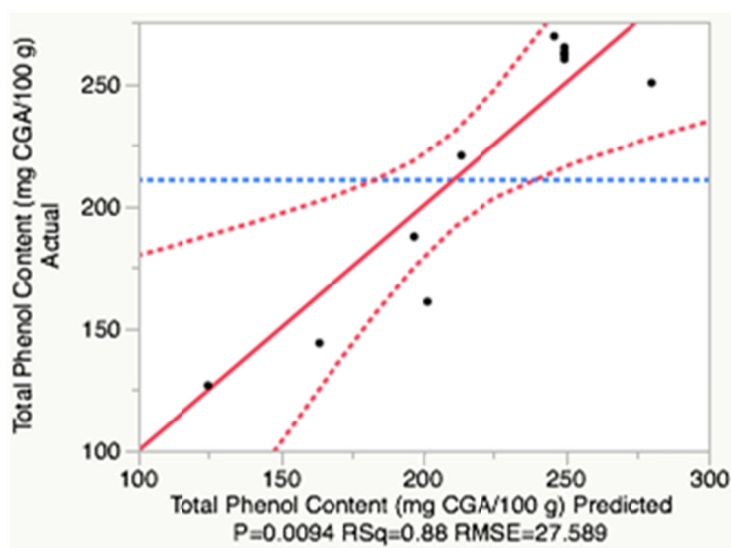
(b)

Figure 2. (a) The response surface for the total phenol content (mg CGA/100 g db) of amaranth grain and (b) the leverage plot based on the ANOVA analysis

Similar procedures were performed for the DPPH and ABTS activities. The response surfaces were constructed and the linear, quadratic, and bi-linear effects of the germination factors were analyzed. The results are presented in Table 4. For all quantities, germination duration (Time) and square of germination temperature (Temp×Temp), the results are significant.

Table 4. Summary of the ANOVA analysis for total phenol, DPPH and ABTS of amaranth grain

	Total Phenol	DPPH	ABTS
Intercept	**249.7216[a]**	**102.6475[a]**	**116.985[a]**
Temp	2.3183	7.5916	1.9716
Time	**58.3483[d]**	**14.605[f]**	**17.8883[c]**
Time×Temp	14.1425	7.2125	4.6375
Temp×Temp	**-50.59[f]**	**-25.2775[f]**	**-21.9[f]**
Time×Time	-27.77	-8.7875	3.37
R²	0.881	0.806	0.805
R²adj	0.783	0.645	0.643
RMS	27.59	13.31	11.59

a: $p<0.0001$,   b: $p<0.0005$,   c: $p<0.001$,   d: $p<0.005$,   e: $p<0.01$, f: $p<0.05$.

As it was observed from Figure 1 and Table 4, ABTS and DPPH are showing a similar pattern to total phenol. This suggests the presence of a linear correlation between these quantities. Existence of this correlation has been reported in the literature (Chlopicka, Pasko, Gorinstein, Jedryas, & Zagrodzki, 2012; PAŚKO, BARTOŃ, FOŁTA, & GWIŻDŻ, 2007; Paśko et al., 2009). In figure 3, scattered plots of the phenol, DPPH, and ABTS versus each other are presented. Unlike the data of Pasko et al. (2009), the results of native seeds are also included. Appropriate linear correlations with acceptable R^2 values are observed.
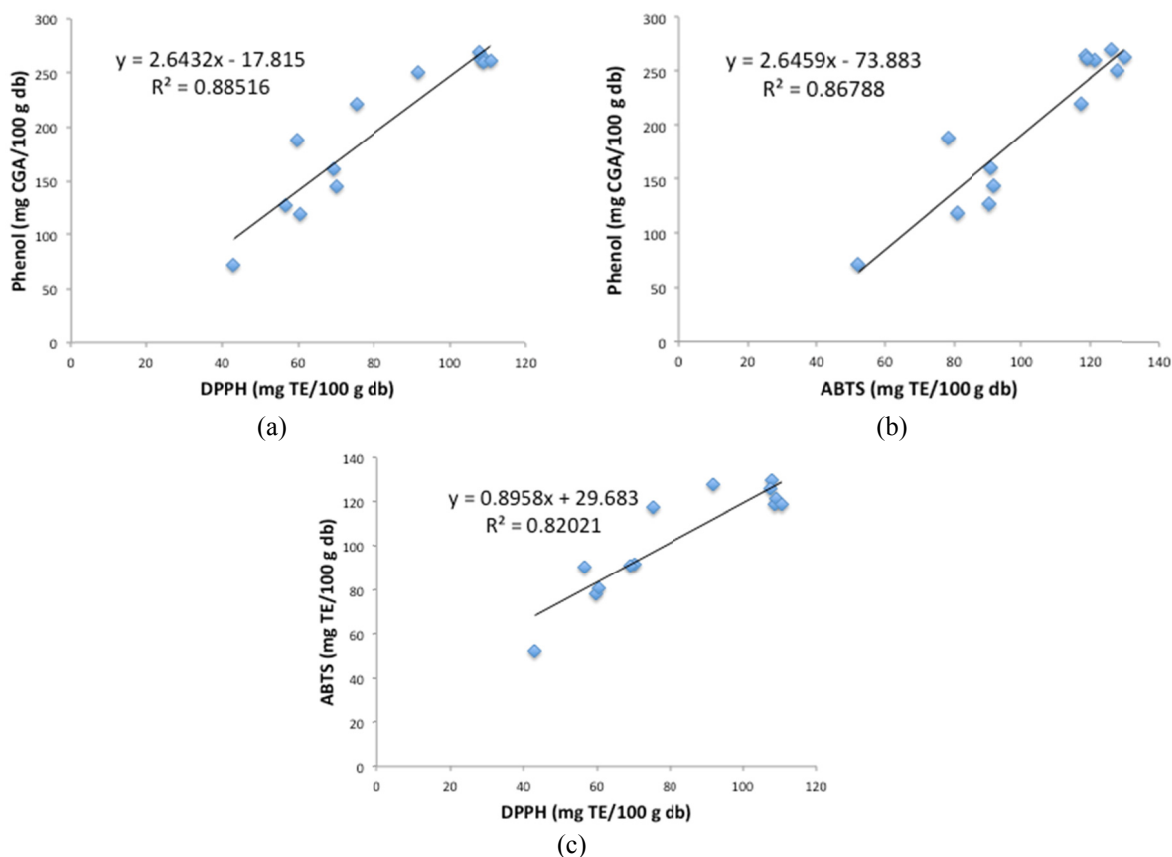

Figure 3. Linear regression curves representing the linear correlations between total phenol content and the antioxidant activities, DPPH and ABTS (a) Phenol vs. DPPH (b) Phenol vs. ABTS, and (c) ABTS vs. DPPH of amaranth seeds

Colorimetric properties of the native and germinated amaranth grains are presented in Table 5. A significant color change (ΔE values) was observed in the amaranth grain during germination, where sprouts' color gradually changed to red. This is clearly observed from the increased a* values shown in the table. The peak of color change occurs in the case of CM6, where grains were germinated for 48 hrs at 26 ℃. This is the case in which phenol and antioxidant activities reached their maximums.

Table 5. Colorimetric properties of native and germinated amaranth grain using CIELab approach

| Exp. # | Germination factors and their levels | | L* | a* | b* | ΔE |
	Temperature	Duration				
GM0	Control		86.98±0.53	0.91± 0.08	12.95± 0.17	0.00±0.56
GM1	22	24	89.44±0.31	0.61± 0.05	11.76± 0.12	2.75±0.34
GM2	22	36	88.16± 0.59	1.64± 0.07	11.75± 0.42	1.84±0.73
GM3	22	48	88.68±0.51	1.19± 0.06	12.20± 0.33	1.88±0.62
GM4	26	24	88.38±0.33	0.83± 0.08	12.15± 0.16	1.61±0.37
GM5.1	26	36	86.19±0.45	3.28± 0.06	11.21± 0.11	3.04±0.46
GM5.2	26	36	86.60±0.75	3.19± 0.06	11.05± 0.17	2.99±0.66
GM5.3	26	36	86.04± 0.64	3.32± 0.17	11.27± 0.49	3.08±0.82
GM5.4	26	36	86.54±0.64	3.18± 0.08	11.16± 0.17	2.92± 0.52
GM6	26	48	83.78±0.70	4.34± 0.12	11.63± 0.44	4.87±0.83
GM7	30	24	88.59±0.43	0.54± 0.09	12.15± 0.33	1.84±0.54
GM8	30	36	88.58±0.61	0.97± 0.05	12.21± 0.23	1.77±0.66
GM9	30	48	85.81±0.77	1.99± 0.09	13.75± 0.27	1.78±0.82

3.2 Millet

A similar experimental procedure and analysis were performed for the finger millet grains. The obtained data are numerically presented in table 6 and graphically in Figure 4. A clear observation from these data is that the germination process decreased the phenol content. The highest decrease was observed for the grains germinated at 26 ℃ for 48 hrs (25% reduction in total phenol content). Besides, germination duration seems to be an important factor, while temperature was not significantly influential.

Table 6. Total phenol (mg CGA/100 g db) content and DPPH (mg TE/100 g db), and ABTS (mg TE/100 g db) activities of finger millet grain for the designed germination treatments

| Exp. # | Germination factors and their levels | | Total Phenol | DPPH | ABTS |
	Temperature	Duration	(mg CGA/100 g db)	(mg TE/100db)	(mg TE/100db)
GM0	Control		627.45±3.93	952.39± 11.30	861.11± 11.10
GM1	22	24	543.29 ±5.87	898.89± 29.28	729.00± 27.29
GM2	22	36	508.60 ± 3.80	829.70± 10.42	643.81± 1.13
GM3	22	48	498.24 ±1.73	812.50± 43.70	657.54± 51.60
GM4	26	24	530.69 ±8.89	794.72 ± 11.43	623.14± 15.69
GM5.1	26	36	480.21 ±11.49	793.22 ± 20.98	587.73± 33.93
GM5.2	26	36	489.85 ±11.59	786.69 ± 14.10	547.57± 25.51
GM5.3	26	36	500.60 ±7.44	808.86± 10.81	621.22± 29.74
GM5.4	26	36	493.76 ±4.99	783.11 ± 92.87	646.71± 26.63
GM6	26	48	466.19 ±10.62	743.11± 24.81	483.11± 21.88
GM7	30	24	515.78±7.99	839.79± 27.30	651.15± 17.53
GM8	30	36	473.59 ±2.78	732.36 ± 41.80	547.09±23.80
GM9	30	48	499.57±0.18	756.54± 58.83	623.05± 25.28
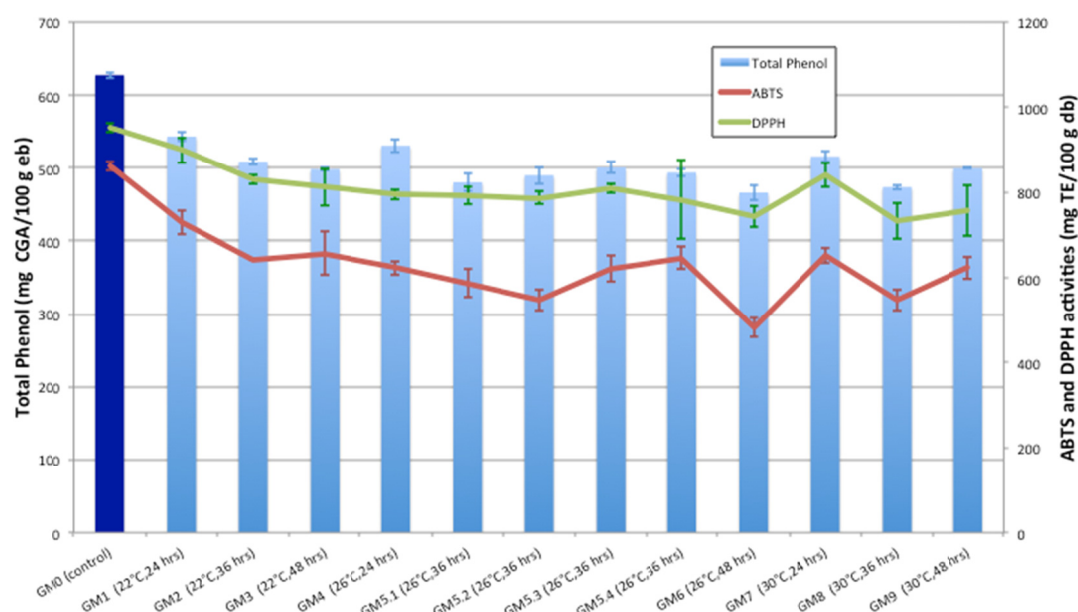
Figure 4. Total Phenol, ABTS, and DPPH activities of finger millet grain for the selected design factor combinations

Table 7. Total phenol content, DPPH, and ABTS antioxidant activities (mg/100 g db) of finger millet grain

Finger millet Variety	Total Phenol (mg/100 g)	DPPH (mg/100 g)	ABTS (mg/100 g)
	Gallic acid equivalent	Trolox equivalent	Trolox equivalent
Brown finger millet (Siwela et al., 2007)	340 to 1840	-	1734 to 4890
White finger millet (Siwela et al., 2007)	<90	-	<1364
Finger millet (Sreeramulu et al., 2009)	373 ±70	173±3	-
Brown color (Chethan & Malleshi, 2007; McDonough, Rooney, & Earp, 1986)	1200 to 2300	-	-
White color (Chethan & Malleshi, 2007)	300 to 500	-	
			Gallic acid equivalent
Millet (Asao & Watanabe, 2010)	360	-	1770
	Catechin equivalent		
Raw Finger millet (Towo et al., 2003)	420 ±27	-	-
Germinated (Towo et al., 2003)	330 ±11	-	-
	Chlorogenic acid equivalent	Percentage basis	
Finger millet	1050	81.67 ± 2.36%	-
Indian Brown (Shankara, 1991)	60 to 670	-	-
Indian Brown (raw) (Sripriya et al., 1996)	102	-	-
Indian Brown (germinated) (Sripriya et al., 1996)	67	-	-
Indian White (Sripriya et al., 1996)	3.47	-	-
Indian Brown (McDonough et al., 1986)	550 to 590	-	-
Indian White (Geetha, Virupaksha, & Shadaksharaswamy, 1977)	80 to 90	-	-
Indian Brown (Geetha et al., 1977)	370 to 960	-	-
African Brown (Geetha et al., 1977)	540 to 2440	-	-
Finger millet (Raghavendra Rao, Nagasampige, & Ravikiran, 2011)	7200 ±570	-	-
	Tannic acid equivalent		
Indian Brown (Shankara, 1991)	30 to 570	-	-
	Ferulic acid equivalents	Ferulic acid equivalents	
Cooked finger millet (Chandrasekara & Shahidi, 2012)	233±4	314.6± 3.5	-

The reported phenol content and antioxidant activities of finger millet in the literature are summarized in Table 8, all in mg/100 g db with respect to different employed equivalents.

Table 8. Summary of the ANOVA analysis of the responses for total phenol content and the corresponding parameter estimates of the different terms of finger millet grain

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	488.4733	5.6711	86.13	**<.0001**
Temperature	-10.1983	5.0724	-2.01	0.0911
Duration	-20.96	5.0724	-4.13	**0.0061**
Duration×Temperature	7.21	6.2124	1.16	0.2899
Temperature×Temperature	7.885	7.6086	1.04	0.3400
Duration×Duration	15.23	7.6086	2.00	0.0922

It is reported that the phenol content in finger millet strongly depends to its color and its geographical cultivation location (Siwela et al., 2007). Generally, white finger millet has lower phenol contents, while brown finger millet is considerably rich in phenolic compounds. The results of our investigated native Indian brown finger millet are completely in range with other reports (Towo et al., 2003). Beside, a similar decrease in the finger millet total phenol content throughout germination process is pointed out in a few other studies (Towo et al., 2003). Towo et al. reported 21% reduction in total phenol after germination of finger millet (Towo et al., 2003). A 34% reduction was reported by Sripriya et al. in germination of Indian brown finger millet (Sripriya et al., 1996). Abdelrahaman et al. reported a 35–42% decrease in polyphenol content of millet germinated for six days in several studied varieties (Abdelrahaman et al., 2007). In this study, phenolic content data were analyzed for finger millet using response surface methodology. Results are presented in Table 9, where only germination duration is found to be a significant factor. Based on this table the following response surface is derived;

$$TP = -10.1983\ X_1 - 20.96\ X_2 + 7.885\ X_1^2 + 15.23\ X_2^2 + 7.21\ X_1\ X_2 + 488.4733 \tag{2}$$

Table 9. Summary of the ANOVA analysis of the responses for total phenol, DPPH and ABTS of finger millet

	Total Phenol	DPPH	ABTS
Intercept	488.4733[a]	781.3108[a]	581.2379[a]
Temp	-10.1983	-35.4[c]	-34.8433
Time	-20.96[b]	-36.875[c]	-39.9316
Time×Temp	7.21	0.785	10.84
Temp×Temp	7.885	23.0375	53.3513
Time×Time	15.23	10.9225	11.0263
R^2	0.832	0.799	0.604
R^2adj	0.692	0.632	0.274
RMS	12.424	27.530	54.747

a: p<0.0001,   b: p<0.01, c: p<0.05.

For case of finger millet, in contrast to amaranth, a negative correlation between phenol content and germination duration exists. This means that as germination duration increases, phenol content decreases in the sprouts. Furthermore, having positive coefficients for the quadratic terms indicates the existence of a minimum in the proposed response surface. The constructed surface is plotted in Figure 5, and it aligns with its corresponding leverage plot. There is a minimum for this surface at 27.49 ℃ and 43 hrs with an interpolated phenol content of 480.28 mg CGA/100 g db. In the performed experiments, the minimum obtained value (466.19 mg CGA/100 g db) for phenol content belongs to case GM6 (Table 6), where the grains were germinated for 48 hrs at 26 ℃.
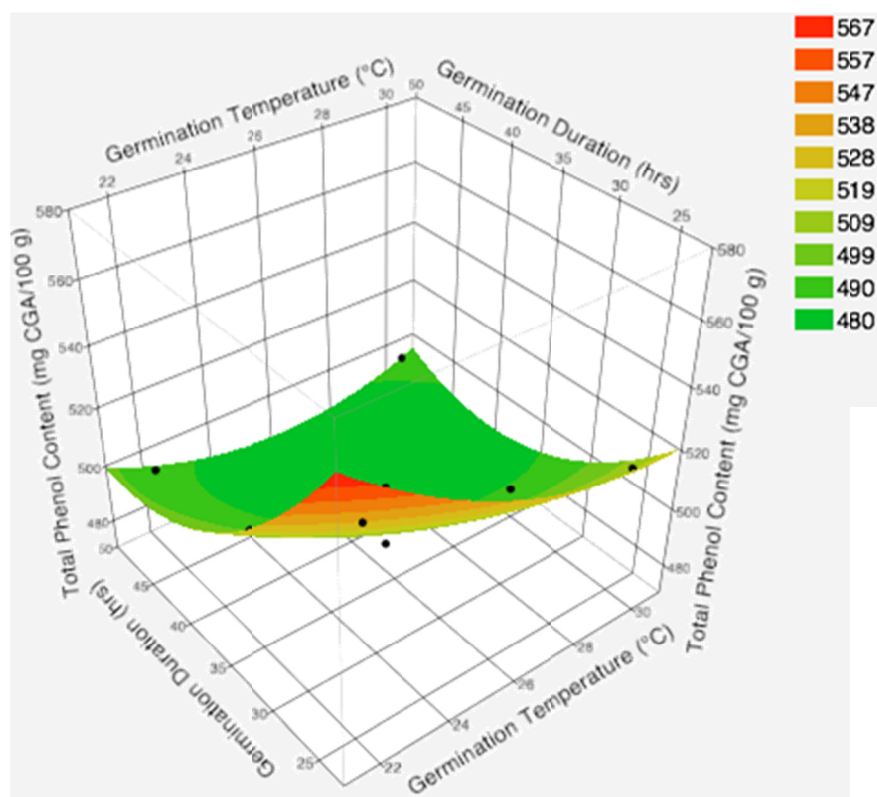
(a)
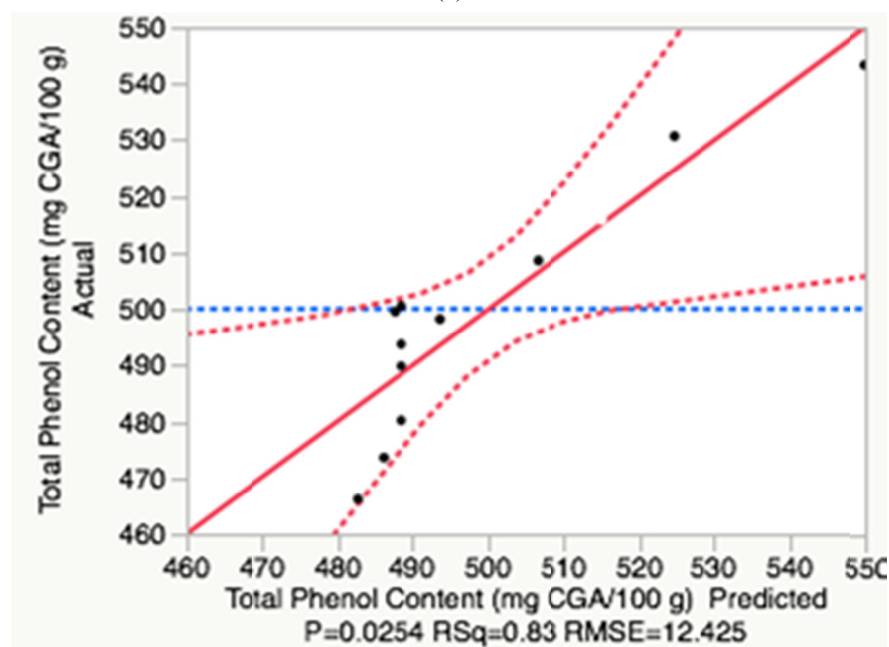
(b)

Figure 5. (a) The response surface for the total phenol content (mg CGA/100 g db) and (b) the leverage plot based on the ANOVA analysis of finger millet grain

DPPH and ABTS activities are similarly analyzed. Results are presented in Table 10. Unlike for phenol content, temperature seems to be an influential factor for the DPPH activities. In addition, despite the proposed response surface for ABTS activity, none of the factors are significantly influential. This is highlighted in the low obtained R^2 value for the ABTS response surface (Table 9).

Table 10. Colorimetric properties of native and germinated finger millet grain using CIELab approach

Exp. #	Germination factors and their levels		L*	a*	b*	E
	Temperature	Duration				
GM0	Control		78.31±0.68	2.77± 0.10	7.23± 0.12	0.00±0.70
GM1	22	24	81.34±0.50	1.82± 0.04	6.50± 0.13	3.26±0.52
GM2	22	36	82.50± 0.51	1.70± 0.05	6.58± 0.15	4.37±0.53
GM3	22	48	82.14±0.56	1.71± 0.07	6.58± 0.14	4.03 ±0.58
GM4	26	24	82.63±0.82	1.73± 0.03	6.39± 0.11	4.52±0.82
GM5.1	26	36	81.98±0.30	1.69± 0.07	6.74± 0.16	3.86± 0.35
GM5.2	26	36	81.91±0.43	1.64± 0.07	6.50± 0.13	3.84±0.46
GM5.3	26	36	81.72± 0.45	1.71± 0.04	6.46± 0.09	3.66±0.46
GM5.4	26	36	81.66±0.49	1.74± 0.10	6.60± 0.07	3.56± 0.51
GM6	26	48	82.16±0.44	1.76± 0.09	6.67± 0.16	4.02±0.48
GM7	30	24	81.47±0.36	1.86± 0.06	6.48± 0.07	3.37±0.37
GM8	30	36	82.11±0.33	1.64± 0.11	6.46± 0.07	4.04± 0.35
GM9	30	48	82.69±0.37	1.81± 0.05	6.79± 0.06	4.50±0.37

Similar to amaranth, possibility of existence of linear correlations between phenol content and antioxidant activities was investigated. In figure 6, the scattered plots of the phenol content, DPPH, and ABTS activities versus each other are presented for native finger millet and its sprouts. Again, acceptable linear correlations were observed, indicating that antioxidant activities decreased as phenol content decreased.
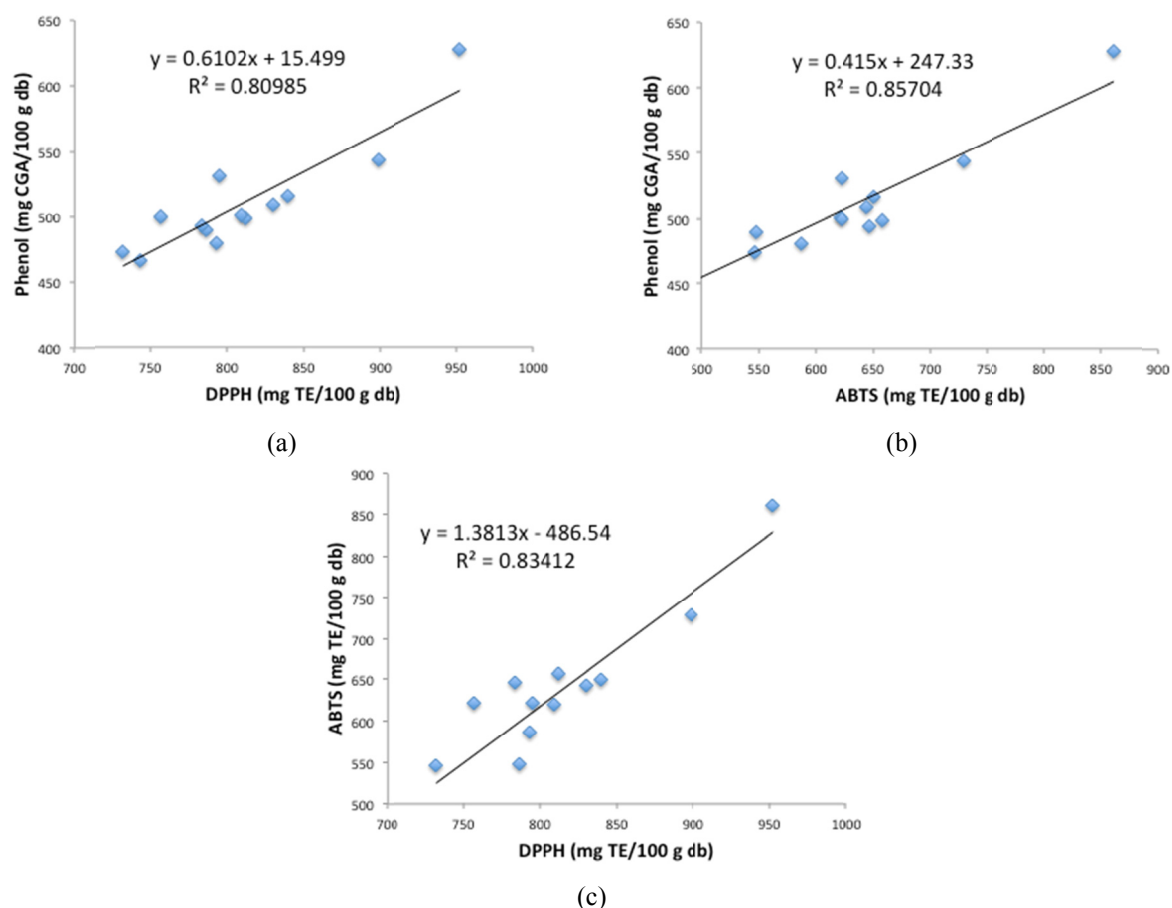

(a)

(b)

(c)

Figure 6. Linear regression curves representing the linear correlations between total phenol content and the antioxidant activities, DPPH and ABTS (a) Phenol vs. DPPH (b) Phenol vs. ABTS, and (c) ABTS vs. DPPH of finger millet grain

Colorimetric values for germinated finger millet grains are presented in table 10, where not a significant pattern was concluded in the color versus germination factors.

4. Conclusion

Finger millet and amaranth grains were demonstrated as two potentially rich sources of phenolic compounds possessing high antioxidant scavenging activities. Malting/germination of these seeds might be an appropriate pre-treatment in the food industry to optimize the phenolic content quantity and quality. In the present study, effects of two important germinating factors, duration and temperature, on the total phenol contents and radical scavenging activities of finger millet and amaranth seeds were investigated. Using a central composite design with three levels for each design factor enabled us to construct a second order response surface for the total phenol content and ABTS and DPPH antioxidant scavenging activities over the interested range of variation (26 ℃<germination temperature<30 ℃ and 24 hrs <germination duration<48 hrs). It was shown that germination significantly (up to four times) increased the phenolic content and antioxidant activities of amaranth seeds. In addition, a significant color change towards pink color was observed in amaranth sprouts, which may increase its consumer acceptability. In case of finger millet, germination slightly decreased the phenol content and DPPH and ABTS activities at most by 25%. Nevertheless, phenolic content of finger millet would still remain appreciably high. For both grains, the extremum point is found to be 26 ℃ and 48 hrs.

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