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Antioxidant and Polyphenol Oxidase Activity of Some Tunisian Pearl Millet (*Pennisetum glaucum* (L.) R.Br.) Ecotypes

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ABSTRACT

Pearl millet is a rich source of various phytochemicals, including phenolic acids. Natural compounds in food are an important health-protecting factor. In Tunisia, many autochthonous pearl millet ecotypes have generated interest because their nutritional qualities. In order to provide information on the composition of pearl millet grown under local conditions, seven ecotypes were analyzed to evaluate their nutraceutical and antioxidant properties. The nutraceutical properties were determined by evaluating the total phenolic while the antioxidant properties were studied using the DPPH free radical scavenging activity. The results showed that the total phenolic varied widely between ecotypes (from 198 to 323 mg GAE/100 g). The DPPH radical scavenging activity of all the ecotypes was relatively high. The antioxidant activity of pearl millet flour significantly varied among ecotypes and ranged from 62.5 to 86.4%. Fractionation of phenolic extracts by HPLC showed 3 major peaks and several minor peaks: *Trans*-cinnamic (486 to 677 µg/g of extract), protocatechic (127 to 452.4 µg/g of extract) and hydroxybenzoic (253.5 to 437.6 µg/g of extract) were the most important. Identifying ecotypes growing under local agricultural conditions with significant levels of beneficial factors could not only provide health benefit to consumers but also promote the value-added cultivation and stimulate industrial and economic growth.

Keywords: authochtonous, polyphenols, phytochemicals, nutraceutical, Tunisia, value added

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br) is one of the important crops in semi-arid areas of Africa and India (Andrews and Kumar 1992; O'Kennedy *et al*. 2006). It is a vitally important cereal for the maintenance of food security in Africa and, together with sorghum, represent about half the total cereal production on the continent and as such are a major source of dietary energy and protein for some 1 billion people in the semi-arid tropics (Rooney 2004; Selton and Taylor 2004). Pearl millet proteins are nutritionally important. As they are gluten-free, they are suitable for coeliacs (Kasarda 2001). Interest in bioactive compounds with antioxidant capacity and potential health benefits in Pearl millet is increasing but data on these antioxidants remains scarce in the literature. Pearl millet, like others cereals, in addition to being primary sources of carbohydrates, also provide trace minerals, dietary fibre and bioactive compounds (Madhujith and Shahidi 2006).

Pearl millet is an important source of some minerals particularly iron and zinc (Serna-Saldivar and Rooney 1995; Adeola and Orban 1995). It has high levels of lipids, high quality and well-balanced proteins (Elyas and El Tinay Yousif 2002). Pearl millet has health-promoting properties, in particular its antioxidant activity (Chung and Pomeranz 1985; Madhujith and Shahidi 2007). It is used as a nutraceutical and as functional food (Dykes and Rooney 2006). Pearl millet has anti-carcinogenic properties (Van Rensburg 1981; Chen *et al*. 1993). It is also a potentially important source of polyphenol and cholesterol-lowering waxes (Hwang *et al*. 2002). It could serve as an important source of phytoceuticals (Awika *et al*. 2003).

Polyphenol compounds can affect a wide range of cell biological functions by virtue of their radical scavenging properties (Aruoma 1993; Rakic *et al*. 2006). Many studies have suggested the role of phenolic compounds as the major source of natural antioxidants in foods of plant origin (Hagerman *et al*. 1998; Balasundram *et al*. 2006). Several workers have highlighted the relationships between food phenol composition and antioxidant activity. While some authors found a correlation between polyphenol content and antioxidant activity, others found no relationship (Moure *et al*. 2001; Heim *et al*. 2002).

Despite the high levels and diversity of phytochemicals in pearl millet, research on this crop as a source of valuable health promoting compounds lags behind other commodities (e.g., fruits and vegetables) and still under researched compared to other cereals. As a result, utilization of pearl millet fractions in foods to improve nutrition is very limited.

Several studies demonstrated that fortification of diet with food components rich in phenolic acids has been shown to impart antimutagenic, antiglycemic and antioxidative properties, and this has been exploited for the development of healthy food formulations (Friedman 1997).

Pearl millet has a big potential, given its agronomic properties, as well as the emerging evidence on the biological effects of the phytochemicals present in the grain.

In Tunisia, pear millet isn't the staple food of rural populations as in the other countries of Africa. Nevertheless, it occupies a very important part of surfaces every year in the centre and in the South of the country (FAO 2003). All pearl millet production is used for a variety of food products. Many autochthonous pearl millet ecotypes have generated interest in Tunisian because their nutritional qualities. However, little information is currently available.

Identifying ecotypes growing under local agricultural conditions with significant levels of beneficial factors could not only provide health benefit to consumers but also pro-

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Table 1 Pearl millet ecotypes surveyed, and their ecological parameters.

Ecotype	Origin	Bio-climatical strata	Annual rainfall (mm)	Annual average temperature (°C)	January average temperature (°C)	July average temperature (°C)
SM	Tataouine	Saharan (Sup)	25-100	22.3	10.5	29.3
KS	Kairouan Kairouan	Arid (Med)	200-400	19.9	11.5	28.7
D - MD- BG	Médnine	Arid (Inf)	100-200	21.3	12.4	26.9
A	Ariana	Semi-arid	400-600	18.3	12.1	26.0
HZ	Mehdia	Arid (Sup)	200-400	19.5	11.9	26.3

Data according to the National Institute of Meteorology (INM), 2009, Tunisia.

mote the value-added cultivation and stimulate industrial and economic growth. Biochemical properties could offer reliable tools to distinguish cultivars or ecotypes and may be considered as criteria of selection for specific food applications. The use of pearl millets not only provides farmers with a market for their products but also saves foreign exchange, which would otherwise be required to import cereals. In fact, food manufacturers as consumers have begun to move towards functional foods with special health effects (Javanmardi *et al*. 2003). Actual studies have focused on genetic improvement of sorghum for biofuel production (Liang 2012) and on genetic variability (iron and zinc content) in diverse sorghum germplasm (Ashok Kumar *et al*. 2012). However, studies regarding antioxidant and nutritional composition are still scarce (Awika *et al*. 2003; Viswanath *et al*. 2009).

In order to provide information on the composition of pearl millet grown under local conditions, seven ecotypes were analyzed to evaluate their phenolic compounds and antioxidant properties.

MATERIALS AND METHODS

Plant material

Seven autochthonous pearl millet ecotypes (*Pennisetum glaucum* (L.) R. Br) from different ecological regions from Tunisia were collected (**Table 1**). Each ecotype is affected of an abbreviation symbolizing the zone of origin.

Plant growth and treatments

Collected grains from each ecotype were sowed at the farm of the Tunisian Agricultural Research Institute in Tunis during the 2007 cropping season. The site is located at 36° 51′ latitude and 10° 11′ longitude. The soil of the experimental site was clay loam. They were sown on 22 May 2007 into randomized block design with four replications. After fructification of the cultivated plants, the grains were homogenously collected for a biochemical investigation. In order to keep relatively uniform sample sizes, we limited our random sampling to 20 plants per ecotype. Equal amounts of grains for each ecotype were pooled and ground to a fine powder to obtain the flour.

Chemicals

Gallic, ferulic, ellagic, vanillic and syringic acids were obtained from Sigma-Aldrich, Chemical Co., Milan. Methanol was of analytical grade and purchased from Carlo Erba (Milan, Italy).

Preparation of polyphenol extracts

About 1 g of flour fractions were refluxed with 1% HCl in CH_3OH (100 ml) for 30 min at 90°C. The extraction was repeated with the residue and the extracts were pooled, neutralised with NaOH, centrifuged (10 min, 3000 rpm), concentrated in a rotary flash evaporator, freeze-dried and stored at 20°C till use (Viswanath *et al*. 2009).

Determination of total phenolic contents (TPC)

Total phenolic compounds from the concentrated extract were determined by the Folin-Ciocalteau micro method (Linkard and Singleton 1977). About 20 µl of extracts solution were mixed with 1.16 ml distilled water and 100 µl of Folin-Ciocalteau reagent, followed after 1 min and before 8 min by 200 µl of Na_2CO_3 solution (20%). Subsequently, the mixture was incubated in a shaking incubator at 40°C for 30 min and its absorbance was measured at 760 nm. Gallic acid (0.1 mg/ml) was used as the standard and the phenolic content in the extract/fraction was expressed as milligram equivalent of gallic acid (GAE) per 100 g flour.

Radical scavenging activity

The antioxidant activity of native and processed little millet extracts were also measured by the DPPH radical scavenging method (De Ancos *et al*. 2002). An aliquot (10 µl) of acidified methanolic extract was mixed with distilled water (90 µl) and 3.9 ml of methanolic 0.1 mM DPPH solution. The mixture was thoroughly vortex-mixed and kept in dark for 30 min. The absorbance was measured at 515 nm. The result was expressed as percentage of inhibition of the DPPH radical. The percentage of inhibition of the DPPH radical was calculated according to the following equation:

% inhibition of DPPH = [Abs control- Abs sample/Abs control] × 100

where Abs control is the absorbance of the DPPH solution without the extracts.

Separation of phenolic compounds

Separation and identification of the polyphenolic components in all extracts was carried out by HPLC (Shimadzu, LC10 A), on a reversed phase chromatography on silica based C18-bonded phase columns (25 cm - 4.6 mm), using a diode array detector operating at different wavelengths (290, 295 and 300 nm). A solvent system comprising of water/methanol (70:30) was used as mobile phase at a flow rate of 1 ml/min. About 100 mg each of freeze dried extracts were dissolved in 1 ml of 50% methanol, 20 µl of each sample was injected into the column. Standards such as gallic, ferulic, ellagic, vanillic and syringic acids were used for identification and quantification of phenolic acids in the samples. Quantification of phenolic acids was carried out by measuring the area under respective peaks and plotting against a standard graph prepared for each individual phenolic acid used (Oneyeneho and Hettiarachchy 1992; Rodríguez-Delgado *et al*. 2002).

Statistical analysis

Quantitative presented data are mean ± standard deviation of triplicate tests. Each experiment was performed in triplicate, and the results were expressed as mean values ± standard deviation. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's test) at 5% level of significance. Values with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The antioxidative phytochemicals in grains, vegetables and fruits have received increased attention recently for their potential role in prevention of human diseases as well as in food quality improvement (Chu *et al*. 2002; Nandita 2002; Nandita and Rajini 2004). Phenolics are considered as a major group of compounds that contribute to the antioxi-

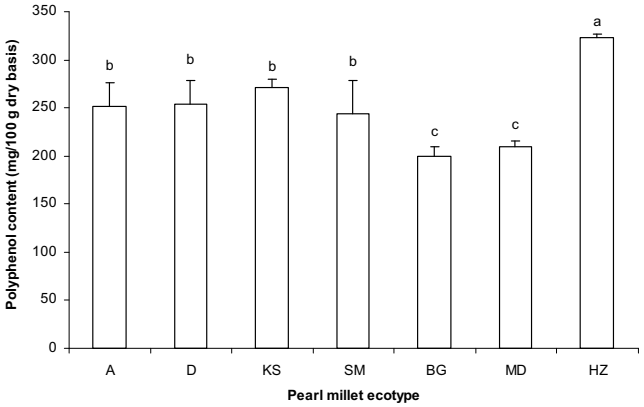

Fig. 1 Total polyphenol content of Tunisian pearl millet ecotypes (A: Ariana, D: Médnine, KS: Kairouan, SM: Tataouine, BG: Médnine, MD: Médnine, HZ: Mehdia). Data are means of three replicates ± standard error (Tukey's test, $P < 0.05$).

dant activities of grains. Pearl millet is an important food grains reported to contain significant quantities of phenolic compounds.

Total phenolic content

Total phenolic content (TPC) in whole pearl millet ecotypes varied significantly among ecotypes (**Fig. 1**). The highest TPC was observed in HZ ecotype with 323 mg/100 g (mg equivalent of gallic acid (GAE)/100 g) while BG showed the lowest TPC (198 mg GAE/100 g).

Phenolic acids are presents in cereal grains (Andlauer and Furst 1998; Towwo *et al*. 2003; Viswanath *et al*. 2009). Their content in cereals is usually less than 1% of dry matter, except for some sorghum cultivars (Subba Rao and Muralikrishna 2002) and finger millet varieties (Chethan and Malleshi 2007a).

The TPC of whole flour from autochthonous pearl millet varied significantly among the ecotypes but it is fairly low compared to many millet varieties cultivated which have polyphenol content of approximately 2.6% (Viswanath *et al*. 2009). Some authors (Sehgal and Kawatra 1998) reported TPC of Bajra ecotype (HHB-50) to be 764 mg/100 g.

Total polyphenols content were 319 and 294 mg/100 g for Composite Pop III and Baladi 1997, respectively (Elyas and El Tinay Youssif 2002). They demonstrated that this in agreement with the fact that polyphenols content of pearl millet is fairly high, as reported by (Alka-Sharma and Kapoor 1996).

Polyphenol quantity and quality in plant foods can vary significantly according to different intrinsic and extrinsic factors such as plant genetics and cultivar, soil composition and growing conditions, maturity state and post harvest conditions, among others (Jaffery *et al.* 2003).

Table 2 Antioxidant properties of pearl millet ecotypes.

Ecotype	% DPPH inhibition
A	70.0 b
D	71.7 b
KS	72.0 b
SM	74.2 b
BG	62.5 c
MD	97.2 c
HZ	96.4 a

Values with same letters (a,b,c,d,e within columns) are not significantly different (Tukey's test, $P < 0.05$).

Antioxidant activity

The DPPH is a stable free radical widely used to determine the antioxidant properties or radical scavenging activity. The radical scavenging capacity against DPPH of all the ecotypes was found relatively high. The antioxidant activity of pearl millet flour significantly varied among ecotypes and ranged from 62.5 to 86.4%. Pearl millet ecotype (HZ) showed the highest antioxidant activity while (BG) ecotype exhibited the lowest (**Table 2**). These results may be due to the presence of highest TPC in the HZ ecotype since the %DPPH inhibition is directly correlated with TPC (Alothman *et al*. 2009). Significant positive correlations between these two parameters were also reported in numerous analytical studies (Dykes *et al*. 2005; Lu *et al*. 2007).

Fractionation of phenolics

The HPLC profile revealed the complex nature of millet polyphenols, and the phenolic constituents were identified using known standards. The phenolic acids reported in pearl millet ecotypes are listed in **Table 3**.

Pearl millet ecotypes vary widely in their phenolic composition and content, with both genetics and environment affecting the kind and level of phenolic compounds.

The HPLC profiles indicated 3 major peaks and several minor peaks revealing the complex nature of pearl millet ecotypes. *Trans*-cinnamic, protocatechic and hydroxybenzoïc are the most abundant. Gallic, ferulic, coumaric, vanillic and others acids were identified and the other components remain to be identified. Further studies are required to characterise these phenolics.

Cereals contain a wide range of phenolic compounds including benzoic and cinammic acids, anthocyanidins, quinones, flavonols, chalcones, flavanones and amino phenolics (Andreasen *et al*. 2000; Adom and Liuy 2002).

The most important phenolic acids in autochthonous pearl millet ecotypes are respectively: *trans*-cinnamic, protocatechic and hydroxybenzoic for all ecotypes. This result is corroborated by (Hahn *et al*. 1983; Waniska and Bandyopadhyay 1989). They found that the phenolic acids in millets are benzoic or cinnamic acid derivatives.

All millets contain phenolic acids, which are located in the pericarp, testa, aleurone layer, and endosperm (Dykes and Rooney 2006). McDonough and Rooney (2000) repor-

Table 3 Phenolic acid composition of different pearl millet ecotype (µg/g of extract).

Phenolic acid	KS	A	BG	HZ	SM	D	MD
Gallic acid	9.8 a	10.9 a	13.6 a	10.1a	11.2 a	7.3 b	9.4 a
Catechin	3.9 b	4.3 b	23.2 a	3.6 b	18.9 a	0.3 d	1.2 c
Ferulic acid	ND	23.6 b	30.2 a	ND	20.3 b	ND	ND
p-Coumaric acid	0.9 a	ND	0.6 a	1.3 a	1.1 a	0.7 a	0.9 a
Gentisic acid	35.4 a	ND	ND	23.3 b	ND	17.3 b	19.7 b
Trans-cinnamic acid	731.2 a	575.8 d	486.3	677.1 b	513.0 e	643.0 c	633.0 c
Protocatechic acid	452.4 a	365.8 c	127.6 d	414.0 b	182.0 d	433.0 b	387.1 c
Hydroxybenzoic acid	437.6 a	253.5 d	ND	391.0 b	ND	382.0 b	352.0 c
Quercitrin	0.7 bc	2.1 a	0.9 b	1.2 b	1.4 b	0.7 c	0.3 d
Quercitin	1.5 b	2.4 a	1.2 b	0.9 c	2.1 a	1.1 b	1.7 b
Vanillic acid	ND	48.3	ND	ND	ND	ND	ND
Caffeic acid	1.3 a	ND	ND	1.7 a	ND	0.9 b	1.3 a

ND = not detected ; Tukey's test, $P < 0.05$

ted the phenolic acid composition of some millet. In 'Finger' and 'Pearl' varieties, ferulic acid was the highest amon all the phenolic acids with 387 and 679.7 mg/100 g dry weight basis. However, in 'foxatil' coumaric acid was the highest with 2133.7 mg/100 g dry weight basis. Phenolic acids consist of two classes: hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids are directly derived from benzoic acid and include gallic, hydroxybenzoic, vanillic, syringic, and protocatechuic acids, among others. The hydroxycinnamic acids include coumaric, caffeic, ferulic, and sinapic acids (Hahn *et al*. 1984; McDonough *et al*. 1986). Phenolic acids such as caffeic, coumaric, ferulic and proto-catechuic acids are also shown to exert an antifungal effect (Aranowski *et al*. 1980).

CONCLUSIONS

Pearl millet is a tropical cereal that has great diversity in its content of phenolic compounds as well as antioxidant activity. These substances are effective biochemical determinants for pearl millet use as food or as source of bioactive components. Research on bioactive components such as phenolic compounds will contribute to unleash the capacity of pearl millet to be a source of nutraceuticals in food formulations and to help to food security in Africa as well as in many developing countries.

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