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Morphological, Phytochemical and Antioxidant Characteristics of White (*Morus alba* L.), Red (*Morus rubra* L.) and Black (*Morus nigra* L.) Mulberry Fruits Grown in Arid Regions of Tunisia

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Abstract - In this study, the morphological characteristics, phytochemical composition and antioxidant activity of white (*Morus alba* L.), red (*Morus rubra* L.) and black (*Morus nigra* L.) mulberry fruits grown in arid regions of Tunisia was investigated. Fruit weight, organic acids, total soluble solids, reducing sugars, total phenolics, total anthocyanins, total flavonoïds, total carotenoïds and total antioxidant capacity (DPPH : 1,1 Diphényl 2 Pycril Hydrazil and ABTS : acide 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonique) were determined. The highest values of fruit weight (2.05 g), reducing sugars (5.02 and 5.75 g 100 g^{-1} fresh weight), total phenolics (30.45 mg Gallic acid equivalent 100 g^{-1} fresh weight) and total anthocyanins (10.05 mg cyanidin-3-glucoside equivalents 100 g^{-1} fresh weight) were measured on *Morus nigra*. The white and the red mulberries have the highest levels of total soluble solids (19.20 %). Regarding malic acid and citric acid content, black mulberry fruits were the less acidic and determined 1.34 and 1.28 g/ l, respectively. Total antioxidant capacity by using DPPH methods of mulberry fruits was found between 66.62 % in *Morus alba* and 71.03 % in *Morus nigra*. The results of the study are helpful for attempting crop improvement in black mulberry for bringing to cultivation.

Keywords: Mulberry, Fruit weight, Organic acids, Reducing sugars, Total phenolics, DPPH, ABTS.

1. Introduction

Mulberry (*Morus spp*. L.) is a perennial tree belonging to the family *Moraceae*. *Morus* includes 68 species (Datta, 2002). Among species, *Morus alba* (white mulberry) originated in southwest China, *Morus rubra* (red mulberry) originated in North America *and Morus nigra* (black mulberry) originated in Iran are wide spread throughout world (Datta 2002; Ercisli 2004; Yilmaz et al. 2012). Mulberry trees are extensively grown in China, India and Brazil for their leaves as food for silkworms, the mulberry cultivated area are 626 000, 280 000 and 38 000 hectares, respectively (Sanchez 2002; Singhal 2009).
Recently, numerous studies have revealed that edible plants are good sources of phytochemicals and play a prominent part in the maintenance of human health (Zafra-Stone et al. 2007; Jiang and Nie 2015). Mulberry have higher contents of polyphenols including flavonoïds, anthocyanins and carotenoïds (Bae and Suh 2007; Lin and Tang 2007; Jiang and Nie 2015). Yilmaz (2011) and Liang et al. (2012) reported that the content of total anthocyanins and total flavonoïds varied widely across different species. The carbohydrates contents of mulberry fruit range from 3 to 30 %. Among them, glucose and fructose are the main sugar (Gundogdu et al. 2011).
In Tunisia, mulberries trees are grown for only fruit production. The fruits can be eaten raw, dried or processed. There are three main mulberry species, namely white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) (Saadaoui and Albouchi 2008). Therefore productivity of trees and fruit characteristics both in quantitative and qualitative aspects is the long-term goal for mulberry breeders in Tunisia. As well known, the genetic improvement of any fruit species depends on the availability of genetic variability in germplasm. Selection of suitable genotypes requires a thorough knowledge on fruit characters and fruit phytochemicals composition of different mulberry species.
A few research efforts on mulberries in Tunisia made so far were restricted to morphological characterize of relevant genotypes from oases regions (Saadaoui and Albouchi 2008). In fact the mulberry improvement program on sound scientific base have just started in the early 2010 in different

research institutes (Arid Land Institute of Médenine- Tunisia), the scientific could able to identify and characterize a lot on accessions in the southeast of Tunisia (Boubaya et al. 2009 and 2011; Thabti et al. 2011). However, Rebai et al. (2012) studied some phytochemical properties of black and white mulberry fruits. A germplasm collection bank with some promising genotypes have been established by the Arid land Institute in El Gordhab, Tataouine in Southern region of Tunisia.
The objective of this study is to compare the morphological characteristics, phytochemical composition and antioxidant properties of white, red and black mulberry fruits in Tunisia, which will provide a basis for planning breeding strategies, fruit processing, as well as selecting accessions with high phyto-nutrient profiles as functional foods.

2. Materials and Methods

2.1. Collection and preparation of mulberry fruit samples

Mulberry fruit were collected from white, red and black mulberry accessions from the germplasm bank established in El Gordhab, Tataouine in Southern region of Tunisia. These genotypes originated from hardwood cuttings and have been established with 4 X 4 m row distances including 3 trees per accession. The collection parcel was installed on sandy soils, cultivated under irrigated conditions and received only the organic fertilization and standard cultural practices. Due to different commercially ripe stage, fruit of the white and black were harvested in the beginning of March 2012 but red fruits were collected in the end of March 2012. The fruits were then transported to laboratory for analysis. Samples were divided into two groups and first group of 40 fruits was used for morphological study (Table 1). The other group (40 fruits) was stored at – 20 °C until further phytochemical analysis.

2.2. Morphological characterization

The assessment of the fruit morphological traits was carried out in 2012 according to the ‘’Mulberry Descriptor ‘’ provided by the Central Sericultural Research and Training Institute (CSRTI 1986). A set of 5 qualitative and 4 quantitative morphological traits were measured on 40 fruits per accession (Table 1).

Table 1. Morphological characters determined in three mulberry species in the arid regions of Tunisia (CSRTI 1986).

Fruit characters	Label	Unit	Explanation
Fruit weight (g)	FW	gr	-
Fruit length (mm)	FL	mm	-
Fruit diameter (mm)	FD	mm	-
Fruit size (length/ diameter)	FS	-	-
Stalk length	SL	mm	-
Fruit color	FC	-	White Brown purple Dark red Black
Density of hairs on skin fruit	SH	-	Sparse Intermediate Dense
Stalk color	SC	-	Light green Green Dark green
Fruit taste	FT	-	Sweet Salty Sour

2.3. determination of pH, total soluble solids, organic acids and reducing sugars of mulberry fruits

Samples of mulberry fruits (40 fruits per accession) were washed drained and squeezed using warning-blender and poured in bottles whose are stored at – 20 °C and used for the analysis. The chemical composition analysis was performed by official methods procedures (AOAC 1995) including pH, total soluble solids (TSS), titratable acidity (malic acid and citric acid). The pH measurements were made using a digital pH meter (Thermo Orion A+, Hong Kong). Total Soluble solids contents were determined by a manual refractometer (OPTECH. MOD. RPU., Germany). The titratable acidity was measured by the citric acid and the malic acid which constitutes the major acids of mulberry fruits. This measure was determined by neutralisation of the total free acidity with a solution of NaOH (0.1 N). Reducing sugars (glucose and fructose) were determined according to the method described by Melgarejo et al. (2000); Gundogdu et al. (2011). Briefly, the samples of 10 g fruit was centrifuged at 12 000 rpm for 2 mn at 4 ° C, then the supernatant was filtered and transferred into a vial and used for analysis. Analysis of reducing sugars was performed by HPLC (KNAUER type) with Eurospher 100 NH2 column and refractive index detector (RI Detectors K-2301) using 80 % acetonitrile as a mobile phase. The calculation of concentrations was based on standards solutions of glucose (2 %) and fructose (2 %). Areas of peaks were determined by the Eurochrome 2000 software. The results were expressed in g 100 g^{-1} FW (fresh weight). All the samples were analysed three times each to calculate mean values.

2.4. Determination of phytochemical composition of mulberry fruits

2.4.1. Determination of total phenolics (TP)

Total phenolics content was measured according to Ercisli an Orhan (2008). procedure. Briefly, fruit slurries of 1.5 g of mulberry were extracted with methanol (60 %) for 3 hours in darkness. Then, extracts were combined with Folin- Ciocalteu's phenol reagent and water, and incubated for 3 min followed by addition of 4 ml of sodium carbonate. After 90 min in darkness, the absorbance at 760 nm was measured by using spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). Samples were replicated three times. Gallic acid was used as standard. The results were expressed as mg GAE 100 g^{-1} FW (fresh weight).

2.4.2. Determination of total anthocyanins (TA)

Total anthocyanins were determined by pH differential method (Giusti and Wrolstad 2005) using a spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). The pigments were extracted by combined 1 g of each sample with 12 ml HCl (1%). After incubation for 48 h at 3 to 5 ° C, the tubes were centrifuged at 4000 rpm. Samples were filtered and stored at – 20 ° C until analysis. Absorbance was measured at 530 nm and 657 nm in buffers ar ph 1.01 and 4.5 using A = [(A530 – A657) pH 1.0 - (A530 – A657) pH 4.5] with a molar extinction coefficient of 29.600. Results were expressed as mg of cyanidin-3-glucoside equivalents (CGE) in g fresh weight (mg cy-3-glue/ 100 g^{-1} FW) basis.

2.4.3. Determination of total flavonoïds (TA)

Total flavonoïds were estimated employing the literature methods involving methanol and Catechin as standard (Djeridane et al. 2006). 1 g of crusher samples was combined with 10 ml methanol. Followed by a filtration and stored at – 20° C until analysed. The sample solution (0.1 ml) was mixed with 0.9 ml distilled water in a tube. Then, 1 ml 2 % AlCl3 was added and mixed. The mixture was allowed to stand for additional 15 mn in darkness. The absorbance was measured at 430 nm using spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). Total flavonoïds contents were calculated using a standard calibration curve, prepared from (+) catechin. The flavonoïds content was expressed as catechin equivalent in mg per g fresh weight of mulberry fruit (mg catechin / 100 g^{-1} FW).

2.4.4. Determination of total carotenoïds (TA)

Total carotenoïds (TC) content was determined according to Nenova (2006) and Aljane and Sdiri (2014). Carotenoïds were extracted by addition 50 mg of each sample with 10 ml d'acétone (80 %). Then, tubes contents were centrifuged at 5000 rpm for for 10 min, and were filtered and stored at – 20 ° C until analysis. The absorbance of each sample was measured at 470 nm, 647 nm and 663 nm using spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). Absorbance (A) was calculated using this formula:

A = [(5 * A470) + (2.846 * A663)] – (14.876 * A647).
Results were expressed as (µg/ 100 g^{-1} FW).

2.5. Determination of total antioxidant properties of mulberry fruits

2.5.1. 1,1 Diphényl 2 Pycril Hydrazil (DPPH) radical-scavenging activity

The DPPH scavenging activity of the extract was measured as described by Rebai et al. (2012) and Bechir Bey et al. (2013). An aliquot (200 µl) of the extract was added to 1 ml of a methanolic DPPH solution (500 µM). The decolorizing process was measured at 517 nm after 30 mn of reaction and compared to a blank control that contained the solvent and DPPH reagent. The scavenging activity percentage of DPPH (%) of the mulberry extract was calculated using this formula: A = (A blank – A sample)/ (A blank) * 100.

2.5.2. Trolox equivalent antioxidant capacity (TEAC)

For the standard TEAC assay, ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in methanolic solution (14 mM) and prepared with 10 ml d'ammonium persulfate (NH2 2S2O8) (4.9 mM) as described by Ozgen et al. (2006) and Ozgen et al. (2009). The mixture was diluted in methanol to an absorbance of 1 ± 0.01 at 734 nm for long stability (Ozgen et al. 2006). For the spectrophotometric assay, 30 µl of mulberry fruit extract and 2.97 ml of $ABTS^{+}$ solution were mixed and incubated for 1 h in darkness. The absorbance was determined at 734 nm using spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). The TEAC was expressed as mg acid ascorbic equivalent per 100 g fresh weight of mulberry fruit (mg AAE 100 g^{-1} FW).

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated by Duncan multiple range test at $P<0.05$ significant level using IBM SPSS Statistics 20.0 software (IBM SPSS Statistics 2011).

3. Results and discussion

3.1. Morphological mulberry fruit characteristics

In the present study, we revealed a variation in morphological characteristics among 3 mulberry species (*Morus alba*, *Morus rubra* and *Morus nigra***)**. In fact, among mulberry species found in Tunisia, *Morus nigra* is the dominant species in cultivation (Saadaoui and Albouchi 2008). Statistical differences at $P < 0.05$ level, were found in fruit weight, fruit diameter and stalk length of the determined parameters of mulberry fruit. Of the 3 mulberry species, the black mulberry fruits have the highest fruit weight. But, the red mulberry fruits have the highest fruit length, fruit diameter and stalk length. As shown in Table 2, fruit color, stalk color, Skin hairs density and fruit taste presented a variation within the 3 mulberry species (Table 2).

In this work, our morphological results were comparable to previous studies. Koyuncu (2004) and Orhan and Ercisli (2010), determined fruit weight, fruit diameter and fruit length were 3.11-4.49 g, 15.34-16.29 mm and 22.33-25.15 mm, respectively. The ranges of mulberry accessions of Orhan and Ercisli (2010) for fruit weight, fruit length and fruit width were 1.77-5.67 g, 20.22-30.49 mm and 11.64-19.42 mm, respectively. The variation revealed for some of the fruits characteristics; however, this is not surprising when considered possible genotypic. Higher fruit weight along with higher yield capacity is the most important desirable fruit characteristic in mulberry breeding programmes (Ercisli 2004; Orhan and Ercisli 2010).

Table 2. Fruit qualitative and quantitative characteristics of mulberry species.

Fruit characters	The white mulberry	The red mulberry	The black mulberry	F value	P value
Fruit weight (g)	1,58 ±0.29a	1,81±0.46 ab	2,05±0.45 b	6.714	.002
Fruit length (mm)	21,38±2.10 a	22,82±2.85 a	21,54±1.86 a	2.337	.106
Fruit diameter (mm)	13,78 ±1.12a	14,73±1.42 b	13,80±0.84 a	4.473	.016
Fruit size (length/ diameter)	1,56±0.14 a	1,55±0.17 a	1,56±0.10 a	0.19	.982
Stalk length (mm)	6,75±1.77 a	10,26±1.70 c	8,19±1.32 b	24.134	.000
Fruit color	White	Brown purple	Black	-	-
Skin fruit hairs ensity	Intermediate	Sparse	Sparse	-	-
Stalk color	Green	Green	Light green	-	-
Fruit taste	Salty	Sweet	Sweet	-	-

0.01, 0.000: significant, high significant at $p < 0.05$ respectively.
Values in the same line with different lower-case letters are significantly different at $p< 0.05$ according to Duncan's test.

3.2. Organic acid and sugars contents in mulberry fruits

The pH, total soluble solids, organic acids and reducers sugars contents of the three mulberry species are given in Table 3. There were statistical differences in terms of total soluble solids, malic acid, glucose and fructose contents among mulberry species in the present study (Table 3). The average pH values of mulberry fruits ranged between 5.56 (black mulberry) and 6.02 (red mulberry). The total soluble solids were 7.27 % (white mulberry) and 12.20 % (red mulberry). As seen from these results, the most predominant organic acids in these mulberry species were malic acid and citric acid (Gundogdu et al. 2011). It was previously showed that fruit pH, total soluble solids, malic acid and citric acid of mulberry fruits which grown in different agro climatic region of Turkey are between 3.43 and 3.66, 14.30 and 19.35 %, 123 and 218 mg/g, 21 and 41 mg/ g (Ercisli an Orhan 2008). The acidity is an important index to assess quality of fresh fruits, the fruits would be tasted sour with a pH values less than 3.50 (Han et al. 2012; Mikulic-Petkovsek et al. 2012; Jiang and Nie 2015). Therefore, these characteristics give the mulberry fruits a more concentrated flavor and better taste, and thus are suitable for either fresh-eating or fruit production (Jiang and Nie, 2015). However, in black mulberry, organic acids were low than white and red species. The difference between species in terms of organic acid content might be caused by genetic factors as well as cultural practices and ecological factors (temperature, light, humidity, etc.) (Gundogdu et al. 2011).

The fruit reducing sugars (fructose and glucose) of 3 mulberry species (*Morus nigra*, *Morus rubra* and *Morus alba*) harvested in China presented a higher results than our species (Jiang and Nie 2015). As well results of Liang et al. (2012) and Jiang and Nie (2015) were generally close to these studies (reducing sugars ranged from 6.33 to 8.18 %). it is interesting that fruit total sugars (12.2 %) of the black mulberry fruits grown in Turkey are similar to our results (total sugars 10.77 g 100 g^{-1} FW), indicating that the *Morus nigra* is likely to maintain these characteristics even under different growth conditions (Ercisli and Orhan 2007; Jiang and Ni, 2015). It is remarkable that, light colored mulberry species (white mulberry) have low glucose and fructose content than dark colored ones (Table 3) (Gundogdu et al. 2011).

Table 3. Fruit acids and sugar contents of examined mulberry species.

Biochemical trait	The white mulberry (*Morus alba*)	The red mulberry (*Morus rubra*)	The black mulberry (*Morus nigra*)	F value	P value
pH	5.98±0.04a	6.02±0.08a	5.56±0.22a	*0.500*	*0.630*
Malic acid (g/ l)	2.68±0.17b	2.57±127b	1.34±0.34a	*1.857*	*0.023*
Citric acid (g/ l)	2.56±0.16b	2.45±1.21b	1.28±0.32a	*2.600*	*0.154*
Total soluble solids (%)	7.27±0.64a	19.20±1.97c	11.60±1.01b	*73.000*	*0.000*
Glucose (g 100 g^{-1} FW)	2.23±0.93a	4.65±2.85b	5.02±0.40b	*3.042*	*0.012*
Fructose (g 100 g^{-1} FW)	2.30±0.91a	5.21±2.65b	5.75±0.50b	*3.469*	*0.010*
Saccharose (g 100 g^{-1} FW)	None detected	None detected	None detected	-	-

0.01. 0.000: significant, high significant at $p < 0.05$ respectively.
Values in the same line with different lower-case letters are significantly different at $p< 0.05$ according to Duncan's test.
FW: Fresh weight

3.3. Total phenolics (TP), total anthocyanins (TA), total Flavonoïds (TF) and total carotenoïds (TC) contents in mulberry fruits

Phytochemical composition Total phenolics, (TP), total anthocyanins (TA), total Flavonoïds (TF) and total carotenoïds (TC) contents in the three mulberry species are presented in Table 4. These parameters differed statistically ($P < 0.05$) among the assayed species (Table 2).

Among the mulberries investigated in this study, black mulberry had the greatest averages of total phenolics (TP) (30.45 mg GAE 100 g^{-1} FW). The contents of total anthocyanins (TA) in species were found between 1.35 mg CGE 100 g^{-1} FW (white mulberry) and 10.05 mg CGE 100 g^{-1} FW (black mulberry). The range of total Flavonoïds (TF) was 8.04 -19.86 mg Catechin 100 g^{-1} FW. Moreover, *Morus nigra* fruits had the highest TF (19.86 mg Catechin 100 g^{-1} FW), means of *Morus rubra* and *Morus alba* was about the half of black mulberry. The results for total carotenoïds (TC) clearly showed that fruits of mulberry had low contents. Furthermore, the black one had the highest total carotenoïds contents (1.23 µg 100 g^{-1} FW). In this study, mulberries are a rich source of phenolics. However, Ercisli and Orhan (2007), presented low levels in Black mulberry (1422 mg GAE 100^{-1} g) and red mulberry (1135 mg GAE 100^{-1} g) of total phenolics. *Morus nigra* and *Morus rubra* are also low in flavonoïds (276 and 219 mg QE 100^{-1} g fresh mass, respectively) Ercisli and Orhan (2007). The investigated mulberry samples contained similar amount of total anthocyanins (Popescu et al. 2014). Equally to our results, Bae and Suh (2007) and Ozgen et al. (2009), found higher anthocyanin contents in black mulberry. The differences of the black mulberry in terms of these phytochemical compositions (TP, TA, TF and TC) are supported to its genetic derivation as well because all trees found same age and ecological conditions (Ercisli and Orhan 2008).

3.4. Antioxidant capacity (DPPH and ABTS) in mulberry fruits

The total antioxidant capacity of whole fruits extracts of the three mulberry species was quantified by two methods (DPPH and ABTS) and we found significant differences among species by using DPPH methods ($P < 0.05$). The black mulberry showed the highest total antioxidant activity (averaging 71.13 % for DPPH and 18.55 mg 100 g^{-1} FW assays, respectively), whereas, the lowest total antioxidant capacity values were found in *Morus alba* species (66.62 % for DPPH and 16.53 mg 100 g^{-1} FW assays, respectively). Ozgen et al. (2009) determined that *Morus nigra* species had the highest total antioxidant activity, with a range of 6.8 -14.4 umol TE/ g fresh mass by the TEAC method. Similar patterns were observed for FRAP and black mulberry had higher values than red mulberry for FRAP. Ercisli and Orhan (2008), found moderate antioxidant activity in selected black mulberries using a different antioxidant method (Lin and Tang 2007; Ercisli and Orhan 2008). In general, species with black and purple skin colored fruits had higher total antioxidant capacity than those with other colors (Ercisli et al. 2012).

Table 4. Total phenolics (TP), total anthocyanins (TA), total Flavonoïds (TF), total carotenoïds (TC) contents and antioxidant capacity (DPPH and ABTS) of examined mulberry species.

Phytochemical trait	The white mulberry (*Morus alba*)	The red mulberry (*Morus rubra*)	The black mulberry (*Morus nigra*)	F value	P value
Total phenolics (TP) mg GAE100 g^{-1} FW	13.51±2.36a	15.73±3.50a	30.45±4.33b	*22.048*	*0.002*
Total anthocyanins (TA) mg CGE 100 g^{-1} FW	1.35±0.01a	1.53±0.14a	10.05±4.34b	*10.562*	*0.011*
Total flavonoïds (TF) mg Catechin 100 g^{-1} FW	8.99±0.38a	8.04±0.93a	19.86±6.47b	*9.906*	*0.013*
Total Carotenoïds (TC) µg 100 g^{-1} FW	0.57±0.25a	0.80±0.09a	1.23±0.34a	*4.000*	*0.079*
DPPH inhibition %	66.62±8.53a	67.43±8.26a	71.13±6.33b	*0.288*	*0.076*
ABTS mg 100 g^{-1} FW	16.53±1.48a	17.78±1.94a	18.55±1.77a	*1.069*	*0.401*

0.001. 0.000: significant, high significant at $p < 0.05$ respectively.
Values in the same line with different lower-case letters are significantly different at p< 0.05 according to Duncan's test.
DPPH : 1,1 Diphényl 2 Pycril Hydrazil
ABTS : acide 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonique
GAE: Gallic acid equivalent
CGE: cyanidin-3-glucoside equivalents
FW: Fresh weight

4. Conclusion

The fruits of these three mulberry species (white, red and black) showed significant different in some morphological traits, and phytochemical components. *Morus nigra* fruits have the highest fruit weight, reducing sugars, total phenolics, total anthocyanins, total Flavonoïds, total carotenoïds and total antioxidant capacity (DPPH and ABTS). The white and the red mulberries have the highest levels of organic acids and highest amount of total soluble solids, respectively. These variations may be due to different species, genetic potential and maturation period. The results of the study are helpful for understanding the morphological variability and the nutrient compositions and attempting the selection of superior desirable mulberry species for bringing to commercial cultivation.

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