Akademik Ziraat Dergisi 11(2): 207-212 (2022)
ISSN: 2147-6403 e-ISSN: 2618-5881 DOI: http://dx.doi.org/10.29278/azd.1061365

Araştırma
(Research)

Organic acid, phenolic acid and flavonoids of medlar during different maturation stages

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Alınış tarihi: 21 Ocak 2022, Kabul tarihi: 3 Kasım 2022

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Abstract

Objective: Organic acid, phenolic acid and flavonoids of *Mespilus germanica* L. cv.'İstanbul' fruit, which was the first registered medlar cultivar in Türkiye, were quantified during different fruit developmental stages by HPLC.

Materials and Methods: İstanbul' fruit, which was the first registered medlar cultivar in Türkiye used the experiments. Organic acids, phenolic acids and flavonoids were identified by HPLC. Phenolic compounds analysed by spectrophotometer.

Results The organic acids were identified in this order of quantity: malic>tartaric>citric>oxalic>pyruvic>fumaric. Malic acid was the major organic acid in all maturation stages (912.9 µg/ g, 346.8 µg/g, 1006.4 µg/g, respectively) while fumaric acid was the minor one. Caffeic acid was the most abundant of phenolic acids, and catechins were dominant among the flavonoids in medlar fruit. Total phenolic compounds increased with maturation (22.98 mg GAE/100g to 28.7 mg GAE/100g).

Conclusion: Organic acid, phenolic acid and flavonoids of Mespilus germanica L. cv.'İstanbul' fruit, which was the first registered medlar cultivar in Türkiye, were detected during different three maturation stages (Immature, semi-ripe, ripe). Malic, tartaric and citric acids were found major organic acids when caffeic acid was major fenolic acid. They changed as the harvest time. It can be recommened that it is necessary to plan the harvesting period depending onhow medlar is meant to be used. It can be harvested at in immature stage if it is usepharmacological and it can be harvested at ripe stage for fresh fruit.

Keywords: *Mespilus germanica*, edible fruits, harvest time, seconder metabolites

Farklı Olgunlaşma Aşamalarında Muşmulanın Organik Asit, Fenolik Asit ve Flavonoidleri

Öz

Amaç: Bu araştırmanın temel amacı, muşmula meyvesinin (*Mespilus germanica* L. cv'İstanbul') organik asit, fenolik asit ve flavonoidleri üzerine olgunluk safhasının etkisini belirlemektir.

Materyal ve Yöntem: Bitkisel materyal olarak 'İstanbul' muşmula çeşidine ait meyveler kullanılmıştır. Meyveler olgunlaşmamış (ham), yarı olgun ve olgun olmak üzere 3 farklı olgunluk safhasında hasat edilmiştir. Meyvelerin, toplam fenolik bileşiklerine ilave olarak bazı organik asit, fenolik asit ve flavonoid içerikleri belirlenmiştir.

Araştırma Bulguları: Organik asitler sırasıyla; malik> tartarik> sitrik> oksalik> pirüvik> fumarik olarak bulunmuştur. Malik asit tüm olgunlaşma evrelerinde (sırasıyla 912.9 µg/g, 346.8 µg/g, 1006.4 µg/g) ana organik asit iken fumarik asit en az bulunanıdır. Fenolik asitlerden en çok bulunanı kafeik asittir. Muşmula meyvesindeki flavonoidler arasında kateşinler baskındır. Toplam fenolik bileşikler olgunlaşma ile artmıştır (22.98 mg GAE/100g ila 28.7 mg GAE/100g)

Sonuç: Türkiye'de tescilli ilk muşmula çeşidi olan Mespilus germanica L. cv.'İstanbul' meyvesinin organik asit, fenolik asit ve flavonoidleri farklı üç

olgunlaşma evresinde (olgunlaşmamış, yarı olgun, olgun) tespit edilmiştir. Kafeik asit majör fenolik asit iken malik, tartarik ve sitrik asitler majör organik asitler olarak bulunmuş olup hasat zamanına göre değişmişlerdir. Muşmulanın nasıl kullanılacağına bağlı olarak hasat döneminin planlanması önerilebilir. Farmakolojik kullanım için olgunlaşmamış, taze meyve tüketimi için ise olgunluk döneminde hasat edilebilir.

Anahtar kelimeler: *Mespilus germanica*, yenilebilir meyveler, hasat zamanı, sekonder metabolitler

Introduction

Medlar (*Mespilus germanica L.*), the most characteristic deciduous tree of the *Rosaceae* family, is consisted of more than 100 genus and 3000 species (Shulaev et al., 2008), and its edible fruit is extremely rich in antioxidants such as phenolics, flavonoids, and other healthy phytochemicals (Ayaz et al., 2002; Glew et al., 2003; Oszmianski et al., 2007; Aygun and Taşçı, 2013). Minor fruit species such as medlar are valuable substances thanks to the preservation and maintenance of genetic resources, their high resistance to diseases and pests, suitability for production and high nutritional value. It is used as a diuretic in folk medicine in South Europe, Türkiye and Iran. Medlar, a typical climacteric fruit species, is hard at maturity harvest, the fruit flesh becomes darker and the fruit softens when it is ready to eat (Glew et al., 2003; Aygun and Taşçı, 2013; Güçlü and Koyuncu, 2018).

Among the constituents of biological samples, organic acids are of increasing interest because of their role in plant physiology as cofactors, buffering agents, and intermediates of the most important metabolic pathways of carbohydrates, lipids, and proteins (Koyuncu, 2004). Organic acids affect many physiological events (e.g. formation of taste, maturation, etc.) in fruit species and also human health. The organic acids, acting on the taste of fruit, balance the sugar levels and constitute the main ingredient of many compounds. It has been revealed by researches that these fast oxidizing compounds have a significant impact on diet. In addition, some organic acids demonstrate antimicrobial effects (Walker and Famiani, 2018). Phenolic substances increase the resistance of plants or fruits to pathogens. Phenolic substances have a strong antifungal effect thanks to the free radicals and quinone they form. Besides the food industry, the use of phenolic substances in pharmacology is quite wide. In the pharmaceutical industry, especially the antimicrobial properties of phenolic substances are utilized. Phenolic compounds also have a positive effect on human health. They have been used in alternative medicine in cardiovascular diseases and cancer treatments for their antioxidant effect due to free radicals (Cemeroglu, 2007; Gündoğdu et al., 2014). Although there are studies conducting phytochemical analyzes on different medlar genotypes (Hacıseferoğulları et al., 2005; Bostan and Islam, 2007; Gulçin et al., 2011; Nabavi et al., 2011; Ercisli et al., 2012; Durul and Unver, 2016; Yılmaz et al., 2016; İşbilir et al., 2019), according to our literature review; this is the first study in which both the change of organic acids and the change of phenolic substances during different harvest periods of 'İstanbul' cv., which was the one of the only two registered medlar cultivar in Türkiye, were examined together. This research aimed to investigate the organic acid, phenolic acid and flavonoid compositions of 'İstanbul' medlar fruits harvested at three different maturation periods.

Materials and methods

Plant material

The fruit of 'İstanbul' medlar cultivar was picked up at 3 different maturation stages from Fruit Research Institute experimental orchard in Isparta, Türkiye is located at latitude 37° 49' N and longitude 30° 52' S. Harvest periods were planned as phenological stages which were identified before by Atay (2013). The fruits were harvested as Immature (Harvest I), semi-ripe (Harvest II) and ripe (Harvest III). Immature; the entire medlar had light green skin, semi-ripe; the skin was 60% light brown and 40% pale green, ripe stage; the skin color of medlar fruit was completely brown, and inside was completely white (Glew et al., 2003). All fruits were harvested randomly in triplicate at each period and stored at –80°C until use.

Organic acids

Medlar fruits (10g) were extracted with 5 mL methanol (≥99.9%, Sigma) and were homogenized (Ultra-Turrax) at 3500 rpm for 10 min. 0.2 mL of pellet was diluted with 1.8 mL of 0.01 M phosphoric acid [H_3PO_4 (pH:1.5)]. The final mixture was filtered through a 0.45 µm membrane filter before 20 µL injections. Organic acids were analyzed using a Shimadzu class LC VP HPLC system with class LC-VP software, a pump (LC-6AD), and a UV-VIS detector

(SPD-10AV VP). YMC Pack-ODS-AM (250 mm 4.6 mm I.D., 5 mm) columns were used. The flow rate was 0.8 mL min^{-1} and the column temperature was ambient. The mobile phases were prepared in distilled water with 0.05 M H_3PO_4, and adjusted to pH 2.2 with NaOH. The UV detector was set at 210 nm. Quantifications were based on the peak area measurements (Caponio et al., 1999).

Phenolic acids and flavonoids

Medlar fruits were dried in the oven at 60°C for 96 hours. Dried samples were broken into pieces and 10 grams of them were extracted for 30 minutes in an ultrasonic bath with 50 ml methanol containing HCl 1%. The extract was filtered with a Whatman filter paper number 4 and evaporated at 40 ^{0}C. The volume of the liquid remaining in the flask without evaporation was completed to 5 ml with methanol. It was filtered through a 0.45 nm filter and 20 µL of it was injected into the HPLC device.

Separation of phenolics was performed by the modified method of Dragovic (Cosmulescu et al., 2020). Reversed-phase (RP)-HPLC analysis was done using an SCL-10Avp system controller, a SIL-10AD VP autosampler, an LC-10AD VP pump, a DGU-14a degasser, a CTO-10 A vp column heater, and a Diode Array Detector with wavelengths set at 278 nm. The 250 × 4.6 mm i.d. 5 µm column used was filled with Luna Prodigy. The flow rate was 0.8 mL/min, the injection volume was 20 µL, and the column temperature was set at 30°C. For gradient elution, mobile phase A contained 2% acetic acid in water; solvent B contained methanol. The amount of phenolic compounds in the extracts was calculated as µg/g fruit using external calibration curves obtained for each phenolic standard.

Total phenolic content

Total polyphenols were quantified by the Folin-Ciocalteu colorimetric method (Arena et al., 2017) and expressed in mg equivalents of gallic acid (GAE) per 100g of sample. Samples were extracted for 24 h in 30 mL 80% MeOH-H_2O at 4°C. Aliquots (15µL) were adjusted to 500 µL with deionized water, and then 250 µL of 50% of the Folin-Ciocalteu reagent (Sigma-Aldrich) and 1.25 mL of 20% (w/v) aqueous sodium carbonate solution were added. After 40 min of sitting at 24°C, the absorbance at 725 nm was measure (Perkin Elmer Lambda 20).

Statistical Analysis

The experiment was a completely randomized design with 3 replications. Statistical analyses were performed with the General Linear Model using SPSS (V.22; Statistical software, SPSS. Inc., USA). The differences among means were analyzed using Tukey's test to estimate the least significant range between mean values.

Results

The changes of organic acids in medlar during different maturation periods were given in Table 1. Malic acid was the predominant organic acid in all maturation stages. Malic acid constitutes 69% of the total acid amount in the Harvest I and reached its maximum level in Harvest III (1006.4 µg/g). The most abundant organic acids are tartaric and citric acid, respectively after the malic acid. It is considered that such fluctuation in dominant organic acid levels is because medlar is a climacteric fruit. Oxalic, pyruvic, and fumaric acid levels were found to be lower in all three periods compared to others. While the total amount of organic acid was 1318.74 µg/g at Harvest I, it was 588.12 µg/g at Harvest II and 1265.99 µg/g at harvest III.

Table 1. Organic acid composition of medlar at three different maturation stages, µg/g

	Harvest period		
Organic acids	**Harvest I**	**Harvest II**	**Harvest III**
Oxalic	26.60 ± 0.36b[x]	29.3 ± 0.02b	32.5±0. 08a
Tartaric	316.60 ± 0.56a	192.0 ± 0.04c	201.7 ± 0.27b
Pyruvic	3.17 ± 0.01a	2.55 ± 0.00b	1.77 ± 0.00c
Malic	912.90 ± 6.60a	346.8 ± 1.80c	1006.4 ± 15.25a
Citric	58.60 ± 0.44a	17.18 ± 0.81c	23.28 ± 0.10b
Fumaric	0.87 ± 0.04a	0.29 ± 0.00b	0.34 ± 0.00b
Σ	1318.74a	588.12c	1265.99b

Mean± Standart deviation.

[x]Values within same row followed by different letters are significantly different ($p<0.05$).

Phenolic acid, flavonoids, and total phenolic substance content of medlar harvested in different periods were presented in Table 2. Caffeic acid was detected as the dominant phenolic acid. It was very high at the beginning of the maturation (79.8 µg/g). Syringic, *p*-coumaric, ferulic, and *trans*-cinnamic acid were the remaining phenolic acids in medlar fruit. Similar to caffeic acid these also reduced with maturation, and no *trans*-Cinnamic acid was found in the 3rd Harvesting period. In all harvesting periods, the highest levels belonged to flavonoid procatechin (29.75 µg/g), (+)-catechin (26.3 µg/g), (-)-epicatechin (26.65 µg/g) and quercetin, respectively. Flavonoids also decreased with maturation. The total amount of phenolic substance was 22.61 mg GAE/100 g, in the first harvesting period, then it increased by 22% with maturation in the last two periods and reached 28.73 mg GAE/100 g in the last period.

Table 2. Contents of phenolic acids and flavonoids in medlar at different maturation stages

	Harvest period		
Phenolic acids, µg/g	**Harvest I**	**Harvest II**	**Harvest III**
Caffeic	79.8±0.00a[x]	26.8±0.00c	30.1±0.00b
Syringic	3.85±0.04a	2.8±0.00c	3.1±0.00b
p-Coumaric	1.9±0.00a	1.6±0.00b	0.7±0.00c
Ferulic	1.3±0.00a	1.1±0.00b	0.95±0.40c
trans-Cinnamic	0.5±0.00a	0.2±0.00b	N.d.*
Flavonoids, µg/g			
Procatechin	29.75±0.10a	21.85±0.10c	26.6±0.08b
(+)-Catechin	26.3±0.10a	17.95±0.00c	20.4±0.08b
(-)-Epicatechin	26.65±0.10a	14.4±0.00c	20.25±0.2b
Quercetin	1.95±0.00a	0.6±0.00b	0.4±0.00c
Total phenolic content, mg GAE/100 g	22.31±0.00b	28.57±0.10a	28.73±0.00a

Mean± Standart deviation. *Nd: Not detected.

[x]Values within same row followed by different letters are significantly different ($p<0.05$).

Discussion

Since the food quality and the impacts of foods on human health have become a key issue, these new foods have found its own place in the industry in a short time and the studies have focused on the functional foods. A wide range of dietary applications of phytochemicals has been found in commonly cultivated fruits of the *Rosaceae* family. Medlar fruit contains important amounts of flavoring, sugars and organic acids that might play a significant role in its flavor also they are important for medlar's consumption form. The number of researches on the use of medlar in the pharmaceutical industry is rapidly increasing.

In the present study, malic, tartaric and citric acids the major organic acids in medlar and their levels changed significantly during fruit maturation. Malic acid was found 912.90 µg/g (Harvest I); 346.8 µg/g (Harvest II) and 1000.6 µg/g (Harvest III) It was arrived own highest value at Harvest III with 1000.6 µg/g. Our results parallel to previous studies. Cevahir ve Bostan (2021) reported that malic, succinic and citric acid contents of the Medlar genotypes were between 590.5 and 1074.5 mg 100 g–1, 127.0–419.0 mg 100 g^{-1} and 2.0–32.0 mg 100 g^{-1}, respectively. Additionally Cosmulescu et al. (2020), reported that malic acid was predominant with a range of 415.08 mg/100 g FW in medlar. In their study where they examined the change of sugars, amino acids, and organic acids during different harvesting periods in medlar; Glew et al. (2003), found that citric acid, malic acid, and ascorbic acid are dominant organic acids. In parallel with the results obtained in this study, they reported that there were fluctuations in organic acid levels in correlation with maturation. Previous findings on the content of malic acid, succinic acid and citric acid were determined between 428 and 1733 mg 100 g−1 (Akın and Bostan, 2018; Selcuk and Erkan, 2015), Natural antioxidants provide the protection of metabolism against the harmful effects of the free radicals and retard process of chronic disorders, as well as the preventing of oxidative deterioration of lipids in foods due to their phenolic contents and they reported that medlar could be used in pharmacology because of its rich phenolic compounds. Polyphenol contents, especially flavonoids and phenolic acids, are responsible for showing antioxidant characteristics of plants. Polyphenols are ready to give electrons or hydrogen

atoms from their hydroxyl moieties to free radicals due to their conjugated electron systems (İşbilir et al., 2019). In our study, caffeic acid was detected as the dominant phenolic acid.79.8 µg/g (Harvest I), 26.8 µg/g (Harvest II),30.1 µg/g (Harvest III). In parallel with our results, caffeic acid was found to be a major phenolic acid with a tendency to decrease along with maturation in medlar (Gruz et al., 2011). During the maturation period, the decrease of phenolic substances accelerates. Mechanical damages or bruises during this period accelerate the oxidation of phenolic substances, causing enzymatic browning. As a matter of fact, as the medlar approaches to eating maturity, it starts browning. It has also been reported in other studies that medlar was rich in phenolic compounds and antioxidants (Ercişli et al., 2012; Canbay et al., 2015; Akbulut et al., 2016).

Conclusions

There are significant differences in the levels of organic acids, phenolic acids and flavonoids of medlar fruit. It can be recommended that medlar can be harvested at the immature stage. (Harvest I), at the same time, the levels of phenolic compounds and certain organic acids are high and the fruits are not mature yet, for pharmacological use. When it is meant to be used as a nutrient, particularly for cardiovascular health protection, medlar harvested at the ripe stage (Harvest III) when the sour taste has been reduced and its flavor and aroma have improved. Therefore, it is necessary to plan the harvesting period depending on how it is meant to be used with this aspect, our study is of importance in the pharmaceutical industry in practice and terms of its utilization as a nutrient. We also believe that this study will become useful to the researchers, especially the ones working on further research considering the obtained data.

Kültür yoğunlukları standart 0.5 Macfarland bulanıklığına ayarlanarak inokülasyon yapılmıştır. *S. aureus* için Metisilin; *Klebsiella* spp.. ve *E.coli* için Tetrasiklin (Himedia SD031-1CT 10 mcg disk^{-1}) standart antibiyotiği pozitif kontrol. metanol ise negatif kontrol olarak kullanılmıştır

Antimikrobiyal aktivite. standart antibiyotikler ve ekstraktlar emdirilmiş disklerin etrafında mikroorganizmaların üremediği şeffaf zon çapları ölçülerek belirlenmiştir (Şekil 3 ve 4).

En yüksek bitki ekstrakt konsantrasyonu (100 mg mL^{-1}) kullanılarak antimikrobiyal aktivite çalışmaları sürdürülmüştür. Özütlerin herhangi bir inhibitör etkisine rastlanmamıştır (Çizelge 8). Sadece çözücüye bağlı inhibitör etki saptanmıştır.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: SFG, EA; data collection: SFG, EA, FK analysis and interpretation of results: SFG, EA; draft manuscript preparation: SFG, FK. All authors reviewed the results and approved the final version of the manuscript.

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