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Comparative Analysis of Antioxidant and Phenolic Content in Hass and Fuerte Avocado Leaves Using Different Extraction Methods

Hass ve Fuerte Avokado Yapraklarında Farklı Ekstraksiyon Yöntemleri ile Antioksidan ve Fenolik İçeriklerin Karşılaştırmalı Analizi

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Abstract

Aim: In recent years, there has been increasing interest in the potential health benefits and sustainable utilization possibilities of antioxidants and phenolic compounds found in the leaves and seeds of avocado fruit. This study aimed to determine the method that maximizes total antioxidant capacity (TAC), total phenolic content (TPC), and flavonoid content (TFC) from the leaves of two avocado cultivars grown in the Alanya region.

Material and Method: TAC of the species was determined using the DPPH•, ABTS+•, and FRAP methods. For the extraction process, Infusion, Maceration, Soxhlet, and Ultrasound-Assisted Extraction methods were used.

Results: The highest ABTS radical scavenging effect was observed in the Hass type with the infusion method and in Fuerte with the Soxhlet method ($p<0.001$). FRAP values were consistently higher in Fuerte leaf extracts than in Hass across all extraction methods, with the highest ferric-reducing antioxidant power observed in Soxhlet extracts ($p<0.001$). The highest TPC and TFC amounts in both plants were obtained with maceration, Soxhlet, and ultrasound-assisted extracts ($p<0.001$).

Conclusion: These results underscore the necessity for detailed determination of the antioxidant capacities and phenolic compound content of different types of avocado leaves and highlight the importance of conducting further studies on extraction and product preparation for R&D purposes.

Keywords: Avocado, Persea americana, Hass, Fuerte, antioxidant capacity, phenolic content, extraction methods, DPPH, ABTS, FRAP

Öz

Amaç: Son yıllarda, avokado meyvesinin yaprak ve tohumlarında bulunan antioksidanlar ve fenolik bileşiklerin potansiyel sağlık yararları ve sürdürülebilir kullanım olanaklarına yönelik ilgi artmaktadır. Bu çalışma, Alanya bölgesinde yetiştirilen Hass ve Fuerte olmak üzere iki farklı avokado çeşidinin yapraklarından elde edilen toplam antioksidan kapasite (TAK), toplam fenolik içerik (TFİ) ve flavonoid içerik (Fİ) miktarlarını maksimize eden ekstraksiyon yöntemini belirlemeyi amaçlamıştır.

Gereç ve Yöntem: TAK değerleri DPPH•, ABTS+• ve FRAP yöntemleri kullanılarak ölçülmüştür. Ekstraksiyon işlemleri için infüzyon, maserasyon, Soxhlet ve ultrason destekli ekstraksiyon yöntemleri uygulanmıştır.

Bulgular: En yüksek ABTS radikal süpürücü etki, Hass çeşidinde infüzyon yöntemiyle, Fuerte çeşidinde ise Soxhlet yöntemiyle elde edilmiştir ($p<0.001$). FRAP değerleri tüm ekstraksiyon yöntemlerinde Fuerte çeşidinde Hass'a kıyasla daha yüksek bulunmuş olup, en yüksek demir indirgeme kapasitesi Soxhlet ekstraktlarında saptanmıştır ($p<0.001$). Her iki çeşitte de en yüksek TFİ ve Fİ değerleri maserasyon, Soxhlet ve ultrason destekli ekstraktlarda tespit edilmiştir ($p<0.001$).

Sonuç: Elde edilen bulgular, farklı avokado çeşitlerinin yapraklarında antioksidan kapasite ve fenolik bileşik içeriklerinin ayrıntılı olarak belirlenmesi gerekliliğini ortaya koymakta ve Ar-Ge amaçlı ekstraksiyon ile ürün geliştirme çalışmalarının önemini vurgulamaktadır.

Anahtar Kelimeler: Avokado, Persea americana, Hass, Fuerte, antioksidan kapasite, fenolik içerik, ekstraksiyon yöntemleri, DPPH, ABTS, FRAP

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INTRODUCTION

Avocado (*Persea americana Mill.*), a member of the Lauraceae family, is widely grown at high rates and widely available in the fruit market for its nutrient-rich fruits. Among the many cultivars, Hass and Fuerte stand out due to their superior commercial and nutritional value. Hass, characterized by its pebbly skin and higher oil content, dominates global markets, while Fuerte, known for its smooth, thin skin and buttery texture, retains a substantial consumer base. Both cultivars are not only valued for their fruit but also for their potential health benefits derived from other parts of the plant, such as leaves.[1-3] The exploration of plant leaves for their bioactive compounds has garnered significant attention in recent years.[4,5] Leaves of various plants are known to be rich sources of antioxidants, phenols, and flavonoids—compounds that play crucial roles in combating oxidative stress and promoting health. Antioxidants neutralize free radicals, thereby preventing cellular damage and reducing the risk of chronic diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders. Phenols, with their strong antioxidant properties, contribute to the prevention of oxidative stress-related diseases and possess anti-inflammatory, anticancer, and antimicrobial activities. Flavonoids, a diverse group of phytonutrients, are similarly noted for their antioxidant effects and their ability to modulate key cellular signaling pathways.[6,7]

Given these significant health benefits, there is a growing interest in identifying and utilizing sustainable sources of antioxidants, phenols, and flavonoids in recent years.[8] Avocado leaves, which are often discarded as agricultural waste, represent an untapped resource with considerable potential. By repurposing these leaves, it is possible not only to enhance the economic value of avocado cultivation but also to contribute to environmental sustainability by reducing waste. Moreover, the use of avocado leaves aligns with the principles of sustainable agriculture and bioprospecting. Sustainable agriculture emphasizes the importance of using all parts of a plant to maximize resource efficiency and minimize environmental impact. By focusing on the leaves of Hass and Fuerte avocados, researchers and agriculturalists can promote more comprehensive utilization of the plant, fostering a circular economy within agricultural systems.[9-13] Previous studies on avocado by-products have reported that avocado leaves contain diverse phenolic compounds, including flavonoids and phenolic acids, with profiles strongly influenced by solvent polarity, extraction technique, and post-harvest drying conditions. In particular, solvent composition and drying temperature have been shown to significantly affect phenolic yield and antioxidant performance of avocado leaves.[8,9,12]

We hypothesized that both extraction method and avocado cultivar significantly influence total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (DPPH, ABTS, and FRAP), with potential interaction effects between extraction method and cultivar. This study was planned and carried out with the aim of evaluating the total antioxidant capacity, phenol, and flavonoid content in the leaves of Hass and Fuerte avocado varieties, as well as determining the optimal extraction method.

MATERIAL AND METHOD

This study was designed and conducted between July 2021 and January 2022 with the aim of determining the total antioxidant and phenolic components of Hass (AH) and Fuerte (AF) avocado leaves grown in Alanya. On September 30, 2021, one kilogram of leaves from *Persea americana Mill*. *cv*, *Hass* and *Persea americana Mill*. *cv*, *Fuerte* types of avocado, respectively, were collected from 1500 m^2 in the eastern region of Alanya (Antalya, TURKEY). These leaves were left to dry to a degree where there were no signs of rotting or molding in a well-ventilated environment without direct sunlight exposure. Following the extraction and lyophilization processes, samples obtained were analyzed for their total phenolic and flavonoid contents as well as total antioxidant capacities. Additionally, extract yields were also examined. (**Table 1**).

Table 1. Extract Yields of Avocado Leaves (%)

Plant Name Statement Code	Infusion	Maceration	Soxhlet	Ultrasound-assisted
Persea americana Mill., cv Hass	AHI	AHM	AHS	AHU
	50	15	25	20
Persea americana Mill., cv Fuerte	AFI	AFM	AFS	AFU
	30	16	20	15

Extraction

As part of the study, leaves of both species were collected in September and dried in shaded and ventilated rooms to examine the effects of direct sunlight drying, considering the negative outcomes reported in previous studies conducted on various plants and/or plant leaves. Leaves were air-dried in a shaded, well-ventilated room at 25±2 °C, relative humidity 50–60%, for 7 days until constant weight was achieved. Dried leaves were ground and sieved to obtain a particle size of ≤500 µm.[14-16] Infusion, maceration, Soxhlet, and ultrasound-assisted extraction methods were employed to prepare extracts from each plant material. To prevent high heat from damaging organic materials, all solvents were removed under low pressure at 38°C. Infusion involved preparing a 5% infusion with hot water (distilled water at 90 °C for 15 min) from 10 g of plant material, followed by filtration through filter paper, and subsequent removal of the solvent using a rotavapor. For maceration, 30 g of plant material was macerated with 70% ethanol at room temperature for 24 hours, followed by filtration of the macerate and removal of the solvent using a rotavapor. Soxhlet extraction was performed by subjecting 30 g of plant material to extraction with a hydroethanolic solvent (70% ethanol), selected based on its reported efficiency in extracting both hydrophilic and moderately lipophilic phenolic compounds, using a Soxhlet apparatus for 8 h, followed by solvent removal under reduced pressure using a rotary evaporator.

Ultrasound-assisted extraction was performed by subjecting 30 g of plant material to extraction with 70% ethanol in an ultrasonic bath operating at 40 kHz and 300 W for 30 min, with the bath temperature maintained below 30 °C, followed by solvent removal using a rotary evaporator.

Measurement of Total Antioxidant Capacity

Three different methods were used to measure total antioxidant capacity. The scavenging effects of the extracts on DPPH radicals were measured according to the method described by Gyamfi et al. (1999), adapted for the current laboratory conditions. Five different dilutions were prepared from the samples to ensure accurate measurements. In a 96-well plate, 20 µl of extract solution was mixed with 150 µl of Tris-HCl buffer (50 mM, pH 7.4) and 150 µl of 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) in methanol. Control and positive control containing no extract and the antioxidant activity of a known compound, Butylated Hydroxyanisole (BHA), respectively, were also used. After incubating the samples in the dark at room temperature for 30 minutes, absorbances were read at 517 nm, and the percentage inhibition was calculated. The half-maximal inhibitory concentration (IC50) values were calculated using non-linear regression curves.

The scavenging effects of the extracts on the ABTS+• radical were determined according to the method described by Thaipong et al. (2006)[17], adapted for the current laboratory conditions to allow plate reader measurements. Five different dilutions were prepared from the samples to ensure accurate measurements. The ABTS radical (7 mM) was prepared by incubating ABTS+• in aqueous solution with Potassium persulfate K2S2O8 (2.6 mM) in the dark for 12 hours and adjusting the absorbance to 1.1±0.02 at 734 nm at room temperature. The radical solution (100 µl) was mixed with sample solution (50 µl) in a 96-well plate and incubated at room temperature for 30 minutes. Absorbances were measured at 734 nm, and the inhibition percentages were calculated as Trolox equivalent (TE) using a Trolox calibration curve.

The ferric-reducing antioxidant power (FRAP) of the extracts was determined according to the method described by Guo et al. (2002)[18] at concentrations of 2, 1, and 0.5 mg/mL, with trolox as a reference. Twenty microliters of sample were mixed with 280 µl of FRAP solution (sodium acetate buffer, TPTZ, and Ferric chloride ($FeCl_3$); 10:1:1). The mixture was incubated at 37 °C for 10 minutes. After incubation, absorbances were read against a control at 593 nm. Each sample was tested in triplicate, and the results were calculated as equivalents of reduced $FeSO_4$ using a $FeSO_4 \cdot 7H_2O$ calibration curve. The interpretation of the results according to the method indicates that increasing absorbances correspond to increased reducing power. In all three methods, experiments were conducted in triplicate, and the percentage inhibition values were calculated based on mean values.

Measurement of Total Phenolic and Flavonoid Content

The total phenolic content (TPC) of the extracts was determined using the method described by Saeed et al. (2012)[19], optimized for the current laboratory conditions. Five different dilutions were prepared from the extracts to achieve the appropriate concentration. Subsequently, 150 µl of sample solution and 125 µl of Folin-Ciocalteu reagent were added to a 96-well plate. After incubating the samples in the dark for 5 minutes, 75 µl of 7% Na_2CO_3 solution was added, and the mixture was incubated for 90 minutes. The absorbance of the samples was measured at 725 nm. The total phenolic content was calculated as mg gallic acid equivalent per gram of extract (GAE/g extract) using a calibration curve derived from the absorbance readings of gallic acid standards, as described by Saeed et al. (2012).

The total flavonoid content (TFC) of the extracts was analyzed using the method described by Ribarova et al. (2005)[20], optimized for the current laboratory conditions. Five different dilutions were prepared from the extracts to achieve the appropriate concentration. Then, 100 µl of extract was mixed with 75 µl of 5% $NaNO_2$ solution in a 96-well plate and incubated for 5 minutes. After adding 75 µl of 10% $AlCl_3 \cdot 6H_2O$ solution at the 5th minute, 50 µl of 1 M Sodium hydroxide (NaOH) solution was added to the plates at the 6th minute, and the absorbances were measured immediately at 510 nm. The total flavonoid content of the extracts was calculated as mg catechin equivalent per gram of extract (CE/g extract) using a calibration curve derived from the absorbance readings of catechin standards. All measurements for TPC and TFC were conducted in triplicate, and calculations were based on mean values.

Statistical Analysis

All statistical analyses, including absorbance calculations, calibration curves, and statistical examinations, were performed using GraphPad Prism Software Version 8.0 (La Jolla, CA, USA). All analyses were performed after testing assumptions of normality and homogeneity of variances using the Shapiro–Wilk test and Levene's test, respectively. Results are expressed as mean±standard deviation (S.S.). Data were analyzed using two-way ANOVA to assess the effects of extraction method and cultivar, followed by Tukey's HSD post hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

Firstly, both species had the highest extract yield with infusion, while the lowest was in Hass with maceration and in Fuerte with ultrasound-assisted extraction (**Table 1**). It has been determined that none of the extracts are as effective as the standard calibration analyte, Butylated Hydroxy Anisole (BHA), in the determination of DPPH radical scavenging activity. Additionally, it was found that the DPPH radical scavenging effect of none of the extracts is high enough to calculate in terms of standard antioxidant BHA equivalent (cut-off value for analysis IC50=0.086±0.052 mg/mL). Therefore, instead of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect, the percentages of radical inhibition by the extracts were calculated. When the extracts were compared among themselves, it was determined that the inhibition rate of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is 48% in the leaves of the Fuerte variety in AFM and AFS extracts, and 46% in the AFU extract. It was observed that the inhibition rates in the extracts of the Hass variety leaves were 47% in the AHU, 46% in the AHM, and 46% in the AHS extracts, respectively (**Figure 1**).

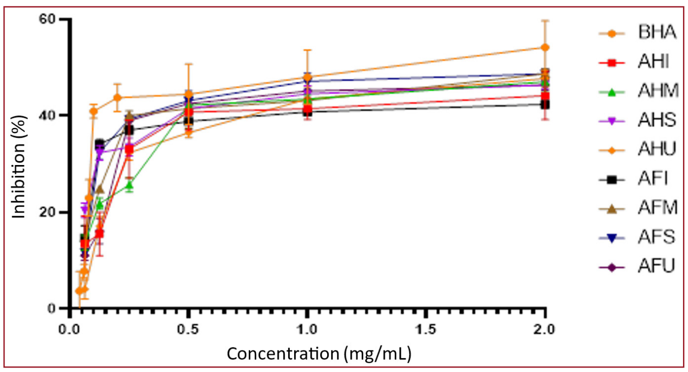

Figure 1. Results of Determination of DPPH Radical Scavenging Effect of Avocado Leaf Extracts
BHA: Butyl Hydroxy Anisole (Calibration Analyte)

When the ABTS radical scavenging effect of the extracts obtained from Hass and Fuerte avocado leaves using an ultrasound-assisted method was found to be similar. The highest ABTS radical scavenging effect was observed with the infusion method in the Hass type and with the Soxhlet method in Fuerte leaves ($p<0.001$). When evaluating the ABTS+• radical scavenging effects of the extracts, the best result was obtained with the infusion of Hass avocado leaf extracts ($p<0.001$). Comparatively, in analyzing the ferric-reducing antioxidant powers of the extracts, the highest result was derived from plants extracted with the Soxhlet method from the Fuerte species ($p<0.001$). Also, the highest antioxidant activity with FRAP among all extracts was observed in the extracts obtained from Fuerte avocado leaves prepared using the Soxhlet extraction method (1369.578±1.789 mmol Fe^{2+}/g extract) ($p<0.001$). When the same type of extracts of Hass and Fuerte leaves were examined, all extract results of Hass leaves gave higher results for the method using ABTS+• radical ($p<0.001$). FRAP analysis revealed significant differences between cultivars and extraction methods ($p<0.001$). Across all extraction methods, Fuerte leaf extracts exhibited significantly higher ferric-reducing antioxidant power than Hass extracts. Among all samples, the highest FRAP value was observed in the Soxhlet extract of Fuerte leaves (1369.58±1.79 mmol Fe^{2+}/g extract), followed by the ultrasound-assisted and maceration extracts. In contrast, Hass leaf extracts showed comparatively lower FRAP values across all extraction methods (**Table 2**). It was measured that there was a statistically significant difference in TPC and TFC amounts in two samples of avocado leaves between infusion, maceration, Soxhlet, and ultrasound-assisted methods ($p<0.001$). The highest amount of TPC and TFC was in the Soxhlet method, while the lowest amount of TPC was in the infusion method and TFC was in the maceration method in the Hass type. In Fuerte avocado leaves, the TFC amount was found to be highest with the maceration method and lowest with the infusion method ($p<0.001$). When the extract results of two different leaves of the same extraction method were examined, Fuerte samples of the extracts obtained by the infusion and soxhlet method gave higher TPC results, and Hass samples of the extracts obtained by the maceration and ultrasound-assisted method gave higher TPC results ($p<0.05$). When the TFC results were compared in the same way, Hass samples obtained by infusion and ultrasound-assisted methods were superior to Fuerte samples obtained by the same method, and Fuerte samples obtained by maceration and soxhlet were superior to Hass samples ($p<0.001$). (**Table 3**).

Table 2. ABTS+• Radical Scavenging Effect and Iron Reducing Antioxidant Power (FRAP) Effects of Hass and Fuerte Avocado Leaf Extracts

Extraction Methods	ABTS (µM Trolox/gextract)					FRAP (mmol Fe^{2+}/gextract)				
	Hass		Fuerte		P	Hass		Fuerte		P
	X±SS	p	X±SS	p		X±SS	p	X±SS	p	
Infusion	0.618±0.002^a		0.545±0.003^d		<0.001	563.800±2.000^g		694.667±3.512^k		<0.001
Maceration	0.555±0.003^b	<0.001	0.586±0.002^e	<0.001	<0.001	474.667±4.042^h	<0.001	840.000±2.000^l	<0.001	<0.001
Soxhlet	0.596±0.002^c		0.614±0.003f		<0.001	510.000±2.000^i		1369.333±2.082^m		<0.001
Ultrasound-assisted	0.615±0.004^a		0.616±0.002f		0.493	864.333±3.055^j		923.333±3.055^n		<0.001

Table 3. Total Phenolic Substance and Flavonoid Content Determination Results of Hass and Fuerte Avocado Leaf Extracts

Extraction Methods	TPC (GAE/gextract)					TFC (CE/gextract)				
	Hass		Fuerte		P	Hass		Fuerte		P
	X±SS	p	X±SS	p		X±SS	p	X±SS	p	
Infusion	20.299±1.107^a		31.691±0.189^d		<0.001	246.177±0.280^h		199.163±0.293^l		<0.001
Maceration	40.633±0.369^b	<0.001	22.150±0.445^f	<0.001	<0.001	237.025±0.113^i	<0.001	354.109±0.103^m	<0.001	<0.001
Soxhlet	65.036±1.088^c		28.266±0.184^g		<0.001	302.089±0.079^j		344.174±0.940^n		<0.001
Ultrasound-assisted	28.531±0.146^d		29.122±0.196^h		0.014	290.913±0.903^k		248.310±0.604^o		<0.001

DISCUSSION

In our study, we prepared a total of eight different extracts from the leaves of Hass and Fuerte's avocado varieties using infusion, maceration, Soxhlet, and ultrasound-assisted extraction methods. The total phenolic and flavonoid contents, along with the total antioxidant levels of the extracts, were measured using DPPH•, ABTS+•, and FRAP assays. The results indicated that the highest total phenolic content was found in the AHS and AHM extracts (65.710±1.088 and 40.514±0.345 mgGAE/g extract, respectively), while the highest flavonoid content was observed in the AFM, AFS, and AHS extracts (354.139±0.104, 344.535±0.902, 302.540±0.088 mgCA/g extract, respectively). Soxhlet extraction was determined to be the most suitable method for both avocado varieties.

Although microwave-assisted extraction is recommended for extracting phenolic compounds in the literature, this method was not preferred in our study due to the potential damage high heat could cause to the plant leaves. A study conducted in Alanya in 2020 found that the Fuerte variety had the highest total phenolic content and was rich in flavonoids.[21] However, our results indicated that the Hass variety contained higher levels of total phenolic compounds. It was hypothesized that the discrepancy between our results and the referenced study might be due to the use of suboptimal extraction methods in the latter.

There is limited research on avocado leaves in the literature. However, studies on the antioxidant and phenolic compound contents of plants like green tea are abundant, and these plants are quite popular for their health claims.[22,23] In 2020, Pacheco-Coello et al.[24] conducted a study in Venezuela examining the phenolic and total flavonoid contents of some commercially available green teas. The results showed that the phenolic contents of the first and second brands were 201.34±3.98 and 178.32±2.22 mgGAE/g product, respectively, while their total flavonoid contents were 118.32±2.09 and 101.43±2.39 mgCA/g product, respectively. Comparing our results to those of green tea, it was observed that avocado leaves have lower phenolic content but are richer in flavonoids. Particularly, Soxhlet and ultrasound-assisted extraction methods showed the highest activity in terms of total antioxidant capacity, surpassing the results for green tea.

In our study, we used three methods widely accepted in the literature to evaluate the total antioxidant capacity. The use of multiple methods has long been recommended due to the varying results they can yield and their potential to provide insights into the further development of plant-derived products.[25] The antioxidant activity results of avocado leaves, when considered alongside the TAC assay results, suggested that the plant is rich in total phenolic and flavonoid contents. The lack of activity in the DPPH system but better results in the ABTS+• and FRAP tests indicated that the plant could be advantageously evaluated in both hydrophilic and classic hydrophobic antioxidant systems due to the nature of these tests. These findings suggest that avocado leaves could be developed as a dietary product and evaluated as a natural antioxidant agent.

Plant leaves, as an important raw material, have played a significant role in medicine and nutrition for many years.[26,27] Many plant leaves, with their high phenolic compound content, have even attracted the interest of numerous scientists (Biglari et al., 2008; Vayalil, 2002). Although the effects of elements such as TAC and TPC intake levels on health are not yet fully clear, the high antioxidant capacity of phenolics obtained from plants is considered to have valuable application potential.[28] Moreover, utilizing the leaves of many cultivated plants, which are normally discarded, as tea, extracts, or dietary supplements—similar to avocado fruit production—holds great importance for both the economy and sustainability.[29] Therefore, developing methods for the extraction, separation, and characterization of phenolic compounds, and evaluating their nutritional values, is highly valuable. The most common extraction methods are traditional techniques like infusion and maceration, frequently used in many studies in this field. Researchers often employ other techniques like ultrasonic-assisted extraction, Soxhlet extraction, or enzymatic methods to enhance the efficiency of solvent extraction, with these methods often proving superior to classical maceration.[30] In our study, Soxhlet and ultrasound-assisted extraction methods outperformed classical infusion and the maceration methods in all experiments except the TAC application with the ABTS radical, especially maceration method, which gave weaker results. Our research highlighted the importance of the extraction method in obtaining antioxidants and phenolics from plant leaves, aligning largely with the literature. Considering the similarity of infusion and maceration methods to traditional food/plant preparation methods (e.g., brewing, winemaking) used by the public, our results suggest the need for more advanced food engineering technologies and the appropriateness of supplement formulations for producing antioxidant and phenolic compound- rich products from avocado leaves.

CONCLUSION

The findings of this study indicate that avocado leaves are a rich source of antioxidant activity and phenolic compounds, exhibiting levels comparable to those reported for several plant leaves in the literatüre. The extract with the highest extract yield was the infusion extract of Hass avocado leaves, and the most bioactive extracts in terms of antioxidant capacity were the soxhelet and ultrasound-assisted extracts of Fuerte leaves and the ultrasound-assisted extraction of Hass leaves. When the results were examined in terms of TPC and TFC, maceration, soxhelet, and ultrasound-assisted extraction extracts came to the fore in both plants. The complexity of the results, taking into account the extract yields, emphasized the need for detailed content determinations in avocado leaves and the need to carry out studies on extraction and product preparation for the R&D product.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study has not required ethics committee approval, and by its nature, the materials and data obtained from this study have not been made available for use.

Informed Consent: Written informed consent was obtained from all participants prior to enrollment in the study..

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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