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COMPARATIVE EVALUATION OF DIFFERENT EXTRACTION METHODS FOR PHYTOCHEMICAL CONTENT AND ELUCIDATION OF MICROSTRUCTURE FROM *MORINGA OLEIFERA* LAM

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ABSTRACT. In this study, the effects of three different extraction methods on the antioxidant capacity, phenolic component, volatile organic molecule, and amino acid contents in the leaves of the Moringa (*Moringa oleifera* Lam.) were compared. The amino acid contents were evaluated via liquid chromatography-diode array detection (HPLC-DAD) analysis. In gas chromatography-mass spectrometry (GC-MS) analysis, loliolide and phytol molecules were detected in moringa leaves. Gallic acid, chlorogenic acid, 4-hydroxybenzoic acid, acid, vanillic acid, p-coumaric acid, hesperidin, hyperoside, quercetin, kaempferol were found in high amounts in moringa leaves by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Tyrosine, glutamic acid, alanine, tryptophan, and L-theanine amino acids were determined by HPLC-DAD analysis. Microstructures images of three extracts were shown with Scanning Electron Microscope (SEM). Element content and mapping were demonstrated by Energy Dispersive X-ray Spectroscopy (EDX). It was found that the best extraction method is MDAE when compared to UBAE and HAE.

Keywords: *Extraction, phenolic compound, amino acid, HPLC-DAD, LC-MS/MS, SEM-EDX.*

INTRODUCTION

Plants and extracts of various parts of them have been used in various treatments in medicine since ancient times. Due to the medicinal bioactive compounds in their structure, they are consumed in various ways

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to increase the immunity of the body [1,2]. Qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of the appropriate extraction method [3,4]. Extraction is the first step of any medicinal plant study and plays a significant and crucial role in the final result and outcome [5]. With the development of modern chromatographic and spectrometric techniques, the determination of bioactive compounds in plants has become easier. However, the extraction method is also effective in the high-yield results obtained in these analyses [6]. An ideal extraction method; should be simple, cheap, fast, and environmentally friendly, and ensure that the desired component is obtained with high efficiency. Efforts to eliminate the problems encountered in classical extraction such as long extraction time, high cost, high purity solvent requirement, the necessity to evaporate large amounts of solvent, low extraction yield, and thermal degradation of temperature-sensitive components [7] have led to the development of new extraction techniques [8]. Methods such as ultrasound-assisted, enzyme assisted, microwave-assisted, pulsed electric field-assisted, homogenizer assisted, supercritical flow, and pressurized liquid extraction have developed as modern extraction methods. Some of these techniques are called 'green techniques' because they comply with the standards set by Environmental Protection Agency, USA [9]. The basic properties sought in extraction techniques developed today are; the use of more reliable chemicals, energy efficiency design, use of renewable raw materials, prevention of pollution, shortened extraction time, low cost, and prevention of accidents [10].

Moringa oleifera Lam. belongs to the *Moringaceae* family and, to date, it represents one of the most important traditional multipurpose food plants [11]. Moringa plants are native to India and Africa and are commonly grown in tropical and arid regions. Thanks to its drought tolerance, the *M. oleifera* tree has spread to other regions, including tropical and subtropical regions. *M. oleifera* is also considered a highly polytropic plant [12]. The seeds, leaves, roots, and even flowers of this plant are fit for both human and animal consumption. The leaves are, in particular, a good source of protein, vitamins, minerals, b-carotene, and antioxidants and have ever been utilized for dietary and medicinal practices [12]. *M. oleifera* has the highest proportion of essential amino acids and significant quantities of minerals when analysed [13]. Several studies have shown that the bioactive components found in the Moringa plant can be used for different industrial and food applications [14, 15, 16]. Intake of essential nutrients and health-promoting phytochemicals increases with the consumption of this plant in humans [17]. The leaves of this plant are used to treat medical conditions such as HIV/AIDS-related symptoms, bronchitis, ulcers, malaria, and fever [18]. It has been confirmed by research that *Moringa oleifera* L. leaf extracts have antioxidant, anti-

hypoglycemic, anti-hypertension, and anti-cancer activities [19,20]. *Moringa Oleifera* Lam. is one of the magical plants considered in many countries of the world due to its high medicinal properties. However, there is still a lot to unleash the potential of *Moringa Oleifera* Lam. by understanding their phytocomponents and variation in extraction due to solvents, understanding their potential properties, and establishing their applications in various fields.

In this study, moringa leaves have been subjected to extraction, and the bioactive compounds from the leaves have been extracted. Three methods were used: microwave digestion, ultrasonic bath, and homogenizer assisted. Methanol was used as a solvent in all three extraction methods. In the obtained extracts, volatile organic compounds, phenolic, and amino acids were determined by chromatographic methods. Images of three different methanol extracts were taken with Scanning Electron Microscopy. In addition, the mineral content of the extracts was shown in the EDS detector of the SEM device by mapping method and their spectra were determined. The antioxidant contents of the extracts were also analyzed. When all analysis data are compared, it was determined which of these three different extraction methods used was more efficient.

RESULTS AND DISCUSSION

Antioxidant Activity Analysis

Antioxidant activities measured in methanol extract of moringa leaves obtained using ABTS, FRAP, and DPPH assays from a single extract were measured three times to test the reproducibility of the assays. Differences were observed according to the three different extraction methods and the results were shown in **Table 1** as TE/g dry weight. According to the FRAP antioxidant activity results, when the microwave-digestion, ultrasound bath, and homogenizer assisted extraction methods were compared, 48.9, 29.3, and 15.65 TE/g DW results were obtained, respectively. In DPPH activity, 64.5, 47.3, and 41.95 TE/g DW results were obtained in these three extractions, respectively. In ABTS activity analysis, 49.25, 34.35, and 16.7 TE/g DW results were obtained in the three extraction methods, respectively. In light of these data, according to the three-antioxidant tests, the highest antioxidant activity was observed in Microwave digestion-assisted extraction. The lowest antioxidant activity was obtained from homogenizer-assisted extraction. It was observed that there was a correlation between the antioxidant capacities of the extracts and the phenolic component results. It was determined that phenolic compounds with strong radical scavenging effects such as Hesperidin, hyproside, and quercetin, especially in microwave decomposition extraction, were higher

than the other extraction methods. Therefore, it could be concluded that its antioxidant capacity was higher than other extractions. Similar to our results, Sreelatha and Padma (2009) reported that they had high antioxidant activity in *moringa* leaves [19].

Table 1. Antioxidant activity results of three extraction methods of moringa leaves

Extraction methods	FRAP mM TE/g DM	DPPH mM TE/g DM	ABTS mM TE/g DM
MDAE	48.9±0.1***	64.5±0.5**	49.25±1.3***
UBAE	29.3±1.17**	47.35±3.5***	34.35±1.2**
HAE	15.65±0.5**	41.95±0.2**	16.7±0.7**

***: P<0.001, **: P<0.05, *: P<0.01, DM: dry mass

As secondary metabolites, phenolic compounds are widely distributed in fruits and vegetables and are considered the main actors in the antioxidant capacity of plants [21]. In a recent study, Castro-López et al. (2017) showed that the extraction method had a strong effect on the recovery of polyphenols from *M. oleifera* leaves [22]. It was determined that the recovery of phenolic compounds was higher especially in the microwave and ultrasonic bath extractions compared to the conventional extraction method, thus corroborating our findings. During microwave extraction, the temperature/microwave energy combination is considered to burst the cell wall, releasing the polyphenols into the extraction solvent more effectively [23].

Phenolic compound identification by LC-MS/MS

The high amount of phenolic component results was given according to MDAE, UBAE and HAE extraction methods, respectively. Gallic acid 9.48, 6.31, 17.47 µg/g DW; protocatechuic acid 22.23, 15.17 and 16.91 µg/g DW; 4-hydroxybenzoic acid 25.61, 21.58, and 21.80 µg/g DW; caffeic acid 21.71, 15.58 and 20.10 µg/g DW; vanillic acid 5.80, 4.50 and 7.25 µg/g DW; p-coumaric acid 12.13, 8.53, and 8,99 µg/g DW; quercetin 24.83, 17.19, and 18.66 µg/g DW; kaempferol 3.82, 3.31, 5.72 µg/g DW were detected (**Table 2**). In particular, the amount of chlorogenic acid, hesperidin, and hyperoside in microwave extraction were 1281.87, 3544.15 and 3070.48 µg/g DW, respectively. Total phenolic component content was obtained as MDAE>UBAE>HAE, respectively. Many researchers have identified the main flavonoids (rutin, hyperoside, kaempferol-3-O-rutinoside, apigenin, quercetin, kaempferol, and D-(+)-catechin) and phenolic acid (chlorogenic acid and rosmarinic acid)

from Italy MOLs extracts [24, 25]. In a study by Fombang et al. (2020), it was shown that the antioxidant activity in alcohol extractions of the leaves of the moringa plant is higher than in other parts of the plant [26]. In this context, Vongsak et al. (2015) reported a strong antioxidant activity for isoquercetin, crypto-chlorogenic acid, and astragalin from *M. oleifera* leaves [27]. Several studies have demonstrated that flavonoid compounds including quercetin, kaempferol, and their derivatives possess remarkable antioxidant activities [28]. Quercetin is a powerful antioxidant that can chelate metals, remove free oxygen slits, and prevent low-density lipoprotein oxidation [29].

Table 2. LC-MS/MS phenolic content of three extraction methods of moringa leaves

Phenolic content (µg/g DW)	**MDAE**	**UBAE**	**HAE**
Gallic acid	9.482±0.09*	6.315±0.10*	17.471±0.22*
Protocatechuic acid	22.23±0.23**	15.17±0.34**	16.908±0.12*
3-Hydroxytyrosol	9.918±0.05*	7.75±0.15*	6.926±0.11*
3.4-Dihydroxyphenylacetic acid	0.950±0.01*	0.878±0.07	0.936±0.08
(+)-Catechin	0.96±0.51	0.34±0.24	0.49±0.23
Chlorogenic acid	1281.87±62.3***	1435.48±73.95***	854.35±61.16***
2.5-Dihydroxybenzoic acid	0.304±0.05	0.105±0.05	0.217±0.03
4-Hydroxybenzoic acid	25.61±0.30**	21.58±0.21**	21.798±0.16**
(-)-Epicatechin	1.15±0.10*	1.66±0.11*	2.59±0.24*
Caffeic acid	21.709±0.08**	15.58±0.14**	20.10±0.13**
Vanillic acid	5.80±1.27*	4.50±5.61*	7.25±8.61*
Syringic acid	0.17±0.17	0.29±0.16	0.41±0.34
3-Hydroxybenzoic acid	0.29±0.00	0.19±0.19	0.10±0.00
Vanillin	1.810±0.09*	1.12±0.09*	1.412±0.0.07*
Taxifolin	0.245±0.02	0.404±0.03	2.682±0.01
p-Coumaric acid	12.128±0.21*	8.530±0.22*	8.987±0.25*
Ferulic acid	2.341±0.27*	1.98±0.18*	2.021±0.06*
Luteolin 7-glucoside	1.606±0.02*	1.359±0.03*	1.77±0.25*
Hesperidin	3544.15±11.5***	3401.65±30.3***	3193.697±40.0***
Hyperoside	3070.476±13.4***	2791.12±18.07***	2574.01±13.0***
Rosmarinic acid	0.65±0.92	2.88±0.48*	1.77±0.11*
Apigenin 7-glucoside	2.378±0.02*	2.139±0.16*	2.454±0.22*
2-Hydroxycinnamic acid	0.16±0.03	0.11±0.02	0.15±0.02
Pinoresinol	1.415±0.17*	1.21±0.25	1.110±0.22*
Eriodictyol	0.042±0.01	0.125±0.05	0.690±0.18
Quercetin	24.830±0.61**	17.19±0.27**	18.66±0.37**
Kaempferol	3.82±0.55*	3.31±0.24*	5.72±0.80*
Total Phenolic	**8046.09±34.3***	**7748.95±42.8***	**6764.62±25.5***

***: P<0.001, **: P<0.05, *: P<0.0

Identification of volatile organic molecules by GC-MS

The results obtained in the GC-MS analysis were defined based on their retention time, percentage of similarity and pattern of mass spectra, and its comparison with the data of the library of NIST11.LIB of the National Institute of Standards and Technology (NIST) and among the identified volatile organic compounds, those with similarities over 80% were shown in **Table 3**. As a result of GC-MS analysis in moringa leaves, Palmitic acid.beta.-monoglyceride, loliolide, and phytol were identified in all three extractions. GC-MS volatile organic compounds results were in agreement with the results of a study by Bhalla et al (2021) [30]. In microwave-assisted extraction and homogenizer-assisted extraction, turmerone was also determined. When the extraction methods were compared, 31, 25, and 21 volatile organic compounds were obtained in MDAE, UBAE, and HAE, respectively. The effective extraction process can be accomplished by undergoing the detection, isolation, and characterization of natural antioxidant compounds. Xu et al., (2017) stated that temperature, time, pH, solvent, and concentration of the sample are the factors that affect the efficiency of the extraction method [31]. Besides, the selectivity of the solvent is expressed depending on the chemical structure and the polarity of extracted antioxidant compounds.

Many methods and techniques have been used to reveal the bioactive components of the Moringa plant. New technologies include methods that offer high-quality, high-yield plant extract, and numerous technical or environmental benefits, such as quick processing times and the use of green solvents, during the extraction procedure. It has been found that green solvents can be ideally used to reveal these compounds [32]. In vitro and in vivo, all parts of the *Moringa oleifera* plant extracted with water, methanol, and ethanol showed excellent antioxidant activity, phenolic activity, antiepileptic, anticonvulsant, antidiabetic, antibacterial, and anticancer activity [33, 34, 35] In a study conducted on the *Moringa oleifera* plant, phenolic compound content was obtained higher in ultrasound bath-assisted extraction than in the conventional extraction method. This resulted in less than 45 min in the extraction process to damage the cell walls with an increase in solvent penetration corresponding to a better phenolic compound yield [36]. In addition, another study reported that microwave-assisted extraction was more efficient than traditional extraction methods in producing polyphenolic compounds from various plants, including Moringa leaves [22].

Table 3. GC-MS results from volatile organic molecules of microwave-digestion assisted, ultrasound-bath assisted and homogeniser assisted extractions. Molecules given in the table were chosen based on 85% or higher similarity

Extraction Methods	Identified Molecule (Similarity%)
MDAE	Acetic acid (97.52); Butyric acid, 4-chloro- (gamma-chloro-n-butyric acid) (90,15); 1,2-Cyclopentanedione (93.81); Oxirane, phenyl-(Styrene oxide) (96.52); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(92.59); 4-vinylphenol; p-vinylphenol (93.17); 2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)- (Carvone) (95.90); Phenol, 5-methyl-2-(1-methylethyl)- (93.86); Benzeneacetonitrile, 4-hydroxy-(90.13); 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (93.03); (-)-Loliolide (92.31); Pentadecanoic acid, 14-methyl-, methyl ester (92.07); Phytol (91.49); diisooctyl-phthalate (93.93); gamma Tocopherol methyl ether (96.49); Propanoic acid, 2-oxo-, methyl ester (methyl pyruvate) (87.59); n-Hexadecanoic acid (Hexadecanoic acid) (89.79); Palmitic acid .beta.-monoglyceride (88.04)
UBAE	Acetic acid (97.52); Butyric acid, 4-chloro- (gamma-chloro-n-butyric acid) (90,15); 1,2-Cyclopentanedione (93.81);4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(92.59); 2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)- (Carvone) (95.90); Phenol, 5-methyl-2-(1-methylethyl)- (93.86); 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (93.03); (-)-Loliolide (92.31); Pentadecanoic acid, 14-methyl-, methyl ester (92.07); Phytol (91.49); gamma Tocopherol methyl ether (96.49); Propanoic acid, 2-oxo-, methyl ester (methyl pyruvate) (87.59); n-Hexadecanoic acid (Hexadecanoic acid) (89.79); Palmitic acid .beta.-monoglyceride (88.04); Tetradecane (85.68); Neophytadiene (85.34)
HAE	Acetic acid (97.52); Butyric acid, 4-chloro- (gamma-chloro-n-butyric acid) (90,15); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(92.59); Phenol, 5-methyl-2-(1-methylethyl)-(93.86); 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (93.03); (-)-Loliolide (92.31); Phytol (91.49); 1,2-Ethanediol, monoacetate (94.1); Propanoic acid, 2-oxo-, methyl ester (methyl pyruvate) (87.59); n-Hexadecanoic acid (Hexadecanoic acid) (89.79); Palmitic acid .beta.-monoglyceride (88.04); Hexadecanoic acid, methyl ester (88.97)

Amino acid content by HPLC-DAD

Fourteen amino acid types in moringa leaves were determined by HPLC-DAD. Especially tyrosine, glutamic acid, alanine tryptophan, and L-theanine were determined in very high amounts (**Table 4**). When three different extraction methods were compared, glutamic acid and alanine contents were obtained as 266.6 and 199.4 µg/g DW, respectively, in microwave

digestion-assisted extraction. In ultrasound bath-assisted extraction, L-theanine and phenylalanine contents were obtained as 130 and 53.8 µg/g DW, respectively. The total amino acid contents when the MDAE, UBAE, and HAE extraction methods were compared, were obtained as 977.2, 939.8, and 680.8 µg/g DW, respectively. Moringa leaves have high amounts of crude protein (23.0 to 30.3%) composed of the essential amino acids methionine, phenylalanine, threonine, leucine, valine, histidine, isoleucine, lysine, tryptophan [37,38,39]. The amino acid contents obtained as a result of three different extractions are in agreement with previous studies. Accordingly, *M. oleifera* leaves are a rich source of essential amino acids, often lacking in many vegetables. For example, the contents of lysine (1325 mg/100 g), phenylalanine (1388 mg/100 g), threonine (1188 mg/100 g), leucine (1950 mg/100 g), and valine (1063 mg/100 g) of the dried leaves are higher than those reported for soybeans and beef [40]. In this study, three different extraction methods were applied to the leaves of the moringa plant. The phytochemical components and antioxidant capacities of the obtained methanol extracts were analysed. When the data were compared, it was determined that the best results were in microwave extraction-assisted, ultrasonic bath-assisted, and homogenizer-assisted extraction, respectively.

Table 4. Amino acid contents of three extraction methods of moringa leaves

Amino Acids (µg/g) DW	**MDAE**	**UBAE**	**HAE**
Aspartic Acid	6.00±0.01*	6.60±0.01*	7.40±0.11*
Glutamic Acid	266.60±0.67***	134.00±0.23***	110.40±0.25***
Asparagine	12.20±0.01**	12.60±0.09 **	6.00±0.07 **
Serine	13.00±0.18**	23.20±0.20**	17.00±0.16**
Glutamine	24.80±0.23**	88.80±0.31***	65.40±0.59***
Arginine	31.40±0.08**	29.40±0.18**	17.00±0.18**
Alanine	127.40±0.53***	126.40±0.14***	73.60±0.14***
Tyrosine	199.40±0.80***	195.20±0.25***	115.00±0.37***
Valine	45.80±0.15***	36.40±0.08***	30.80±0.31***
Tryptophan	50.00±0.47***	33.80±0.15***	33.60±0.23***
Phenyl Aniline	37.80±0.25***	53.80±0.15***	39.20±0.17***
Isoleucine	19.80±0.18**	31.20±0.01**	21.80±0.09**
Leucine	25.60±0.16**	38.40±0.04**	29.60±0.13**
L-Theanine	117.40±0.63***	130.00±0.45***	114.00±0.54***
Total Amino acid	**977.2±0.42**	**939.8±0.28**	**680.8±0.42**

***: P<0.001, **: P<0.05, *: P<0.01

Scanning Electron Microscope – Energy Dispersive X-ray Spectroscopy (SEM-EDX) results

According to the SEM results, the images of the three different extractions are different from each other. SEM images of unextracted (before extraction) moringa leaves were also given in figure 1 (d, d1). The results in microwave digestion-assisted extraction are quite interesting when images are taken at 4 µm magnification for each extraction (**Figure 1**). In microwave, digestion assisted extraction images it was observed that there were quite large divergences in leaf morphology. It has been reported and reviewed that microwave irradiation of plant samples leads to intense structural destruction, shrinking of plant parts and irregularities in the plant surface [41]. In the ultrasonic bath-assisted extraction, the leaf epidermis surface was damaged and wrinkling occurred (**Figures 1b, 1b1**). The ultrasonic waves propagate into the liquid, resulting in alternating high-pressure (compression) and low-pressure (rare fraction) cycles. In the rare fraction half-cycle of ultrasonic waves, a vast amount of small vacuum bubbles was generated by the high-intensity ultrasonic waves [42]. This event was followed by cavitation. Cavitation also results in the formation of high-pressure shock wave and the generation of powerful liquid jet that is expelled at the leaf surface [43]. It was observed that there were breaks and deteriorations in the leaf surface morphology in homogenizer-assisted extraction. It can be said that the reason for this is the high rotational speed of the homogenizer device. We can see that it breaks the leaf epidermis tissue due to its high rotation speed (**Figures 1c, 1c1**). Element mapping was done with Energy Dispersive X-ray Spectroscopy (EDX). Carbon (C), oxygen (O), magnesium (Mg), sulphur (S), chloro (Cl), and potassium (K) elements were observed in all three extraction maps (**Figures 2a, 2b, 2c, 2d**). Element detail analyses by EDX detector results were given in **Figures 3a, 3b,3c,3d**. Elemental analysis of a specific region was performed for each extraction. The amount of minerals obtained was shown as %. Potassium contents were obtained as MDAE, UBAE, and HAE at 2.25%, 1.05%, and 0.60%, respectively. The lowest carbon content of 54.19% was obtained from MDAE.

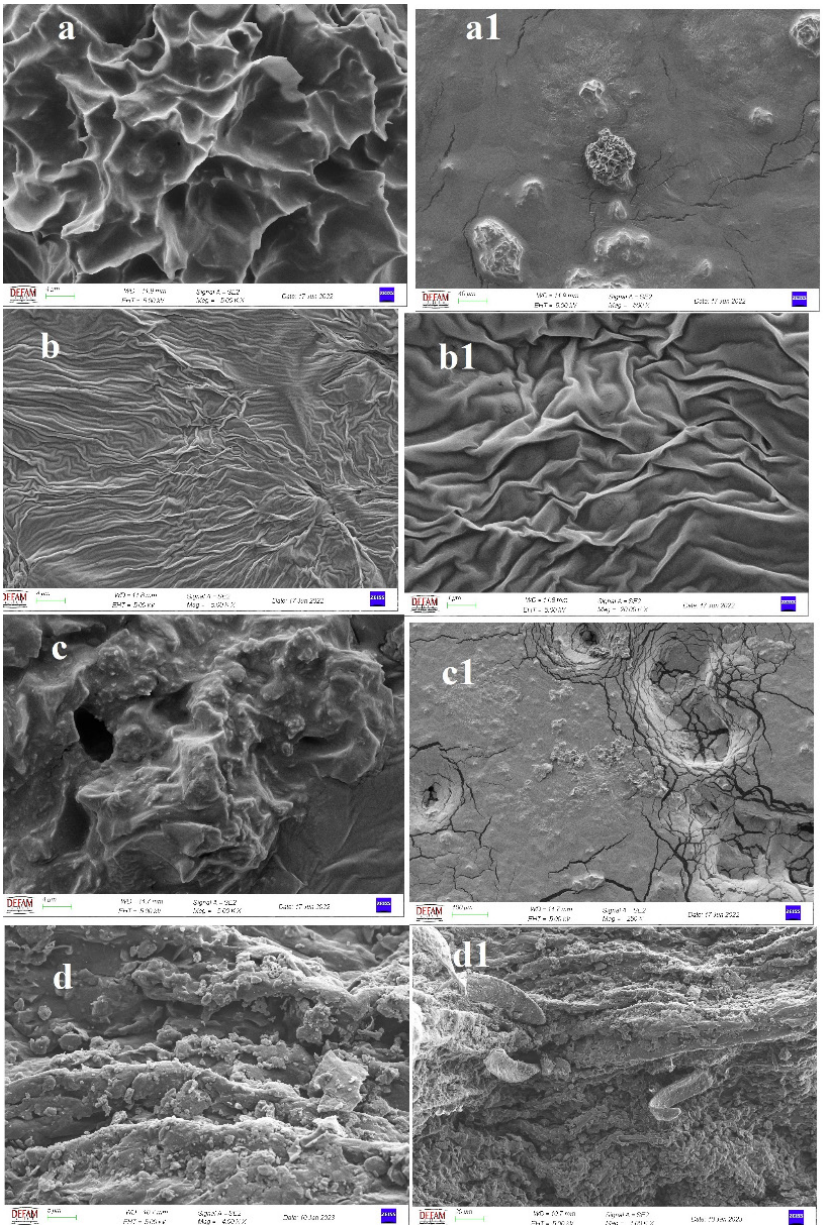

Figure 1. Scanning electron microscope (SEM) images of three plant leaves extractions. a, a1: MDAE, b, b1: UBAE, c, c1: HAE, d, d1: non-extracted/before extraction.

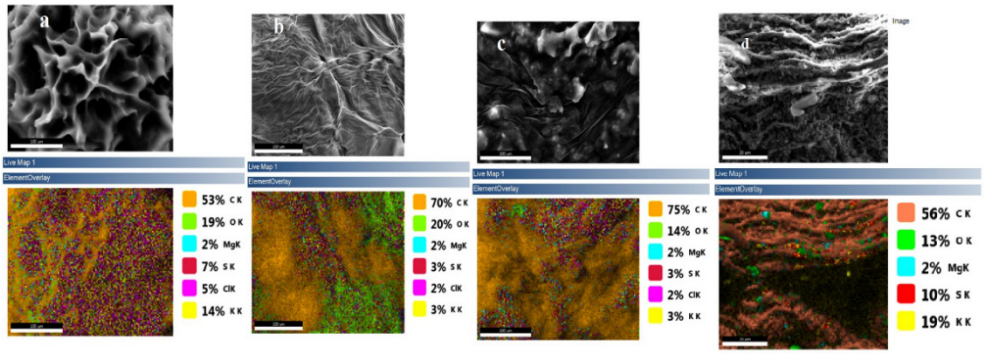

Figure 2. SEM-EDX element mapping results of three plant leaves extractions. a: MDAE, b: UBAE, c: HAE. d: non-extracted/before extraction.

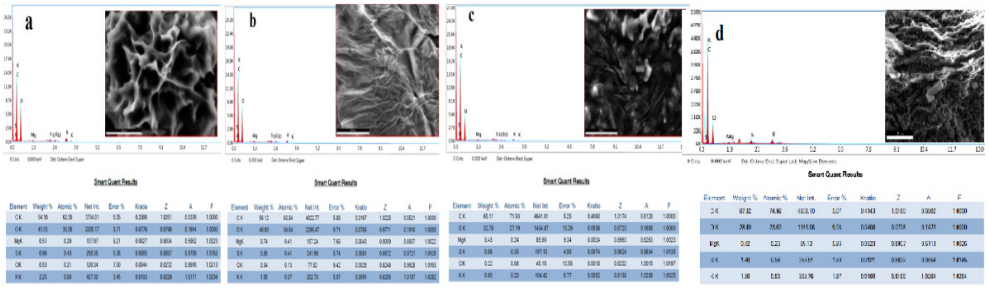

Figure 3. EDX element concentrations (%) of three plant leaves extractions. a: MDAE, b: UBAE, c: HAE, d: non-extracted/before extraction.

CONCLUSION

Extraction is the pre-process that should be done before bioactive component analysis. Today, there are many extraction methods in use. However, it is very difficult to determine an effective extraction method. With an efficient extraction process, product loss, amount of solvent, and wasted time can be minimized. In this study, three different modern extraction methods, microwave decomposition, ultrasonic bath, and homogenizer assisted, were compared. The leaves of *Moringa oleifera*, which is a good source of bioactive compounds, were used as plant material. Phytochemical analyses were carried out with various chromatographic modern devices in the obtained extracts. A detailed determination of the antioxidant capacities and phytochemical

content of moringa leaves after extractions allowed a comparison of extraction efficiency. As a result of three different extractions, differences in phenolic contents and accordingly antioxidant activities were observed. It was found that especially the microwave decomposition method was more effective than other methods and provided the release of phenolic compounds with radical scavenging antioxidant properties. In addition, it has been shown that each extraction creates microstructural differences with SEM images. Thus, it can be said that the selection of the extraction method affects the release of biocomponents as it changes the structure of the plant surface. Especially, a comparison of non-extracted and extracted moringa leaves was demonstrated with SEM images. Specific and distinctive differences were seen in the images obtained. Alanine, glutamic acid, and tyrosine, which are essential organic acids for human health, were determined at high rates in all extractions. However, when the three extractions are compared, it can be said that the effective extraction method is MDAE, UBAE, and HAE, respectively.

The importance of natural products rich in bioactive components is increasing day by day. Therefore, this study may guide similar studies to be conducted in the future.

EXPERIMENTAL SECTION

Chemicals and reagents

Moringa leaves were purchased from an herb market in Manisa city (38.749444°N 28.122778°E), Turkey, in August of 2020. The leaves were washed to remove impurities and then air-dried in the shade before extractions. All standards included chlorogenic acid (>99.0%), D-(+)-catechin (>99.8%), rutin (>99.7%), hyperoside (>99.8%), kaempferol-3-Orutinoside (>99.7%), astragalin (>99.8%), rosmarinic acid (>99.7%), polydatin (>99.8%), quercetin (>99.8%), apigenin (>99.8%), kaempferol (>99.8%) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, Missouri, USA). ABTS (2,2′ -Azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, Missouri, USA). Methanol, ethanol, and acetonitrile (≥99.9%, (for HPLC) was obtained from Merck. Amino acid standards 10×1 mL, Sigma/AAS18, Fmoc chloride (FMOC-Cl) (≥% 99.0) from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, Missouri, USA), OPA (o-phthaldialdehyde) from Alfa Aesar, Thermo Fisher Scientific Chemicals, Germany.

Extraction methods used for moringa leaves

Microwave digestion-assisted extraction (MDAE)

Dried leaves samples of 0.5 g were weighed, and 20 mL of methanol (100%) was added. Extraction was performed in a Microwave digestion device (Cem, Mars 6 version, NC, USA.), setting the temperature to 55 ºC, 15 min ramp, 25 min hold, and 20 min cooling program. The obtained extract solution was filtered. and kept at +4 ºC in amber glass vials until the other analysis.

Homogeniser-assisted extraction (HAE)

Dried leaves samples of 0.5 g were weighed, and 20 mL of methanol (100%) was added. Extraction was performed by using an Ultra-turrax (IKA T25, Staufen, Germany) at 5000×g for 3 min at room temperature. The extracts were then centrifuged (Hettich- universal 320, Tuttlingen, Germany) at 10.000×g for 10 min at 4 °C. Finally, the resulting solutions were collected in amber glass containers until the other analysis.

Ultrasound bath-assisted extraction (UBAE)

Dried leaves samples of 0.5 g were weighed, and 20 mL of methanol (100%) was added. Extraction was performed by using an ultrasonic bath device (Wised, Wisd-WiseClean, Germany), for 30 min at 45 °C. The obtained extract solution was filtered and kept at +4 ºC in amber glass vials until the other analysis. The percentage yield of extraction was calculated as:

Percentage yield = weight of dry extract/weight of dry plant material X 100%.

The percentage yield of microwave digestion-assisted extraction (MDAE): 22.96.
The percentage yield of a homogeniser-assisted extraction (HAE): 17.24.
The percentage yield of ultrasound bath-assisted extraction (UBAE):15.48.

Antioxidant activity assays

The FRAP analysis was performed according to the following procedure with some modifications [44]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20mM $FeCl_3.6H_2O$ solution.

The fresh working solution mix was prepared as follows: 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5mL $FeCl_3.6H_2O$ solution and then warmed at 37 ^{0}C before use. Leaves extracts (150 µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in a dark condition. Then, absorbance was taken at 593 nm using the spectrophotometer (TECAN, Männedorf, Switzerland). The standard curve was linear between 25 and 600 mM Trolox. Results were expressed in mM Trolox equivalents (TE)/g dry mass (DM).

The DPPH analysis was performed according to the following procedure with minor modifications [45]. The stock solution was freshly prepared by dissolving 24 mg of DPPH in 100 mL of methanol, and then 10 mL of this solution was taken and diluted with 45 mL of methanol. Leaves extracts (150 µL) were allowed to react with 2850 µL of the DPPH solution for 2 h in a dark condition. Then, absorbance was taken at 515 nm using the spectrophotometer (TECAN, Männedorf, Switzerland). The standard curve was linear between 25 and 800 mM Trolox. Results are expressed in mM Trolox equivalents (TE)/g dry mass. In all measurements, additional dilution was needed if the analysis value measured was over the linear range of the standard curve.

For ABTS assay of leaf extracts was performed according to the following method with some modifications [46]. A stock solution containing 7.4 mM ABTS and 2.6 mM potassium persulfate was prepared. The prepared stock solution was kept at room temperature for 12 h and then 1 mL was taken and diluted with 60 mL of methanol before the analysis. Leaves extracts (150 µL) were allowed to react with 2850 µL of the ABTS solution for 2 h in a dark condition. Then, absorbance was taken at 734 nm using the spectrophotometer (TECAN, Männedorf, Switzerland). The standard curve was linear between 25 and 600 mM Trolox. Results were expressed in mM Trolox equivalents (TE)/g dry mass.

Determination of phenolic compounds by LC-MS/MS

Determination of phenolic profiles of leaves extracts, high-performance liquid chromatography-mass spectrometer - mass spectrometer (Agilent 1260 Triple Quadrupole MS/MS) were used. Each analysis was performed with three replications. HPLC column C18 ODS used in the analyses (25x4.6 mmx5 µm) was used. Injection volume for analysis: 2 µL. Water/0.1% formic acid (A), and methyl alcohol (99.9%) (B) was used as a carrier phase. The gradient method is as follows: 3 min 2% B, 6 min 25% B, 10 min 50% B, 14 min 95% B, 17.5 min 2% B. Flow rate: 0.4 mL/min. The identification of compounds was performed in positive and negative modes [47]. LC-MS/MS total ion chromatograms of phenolic compounds were shown in **Figure 4**.

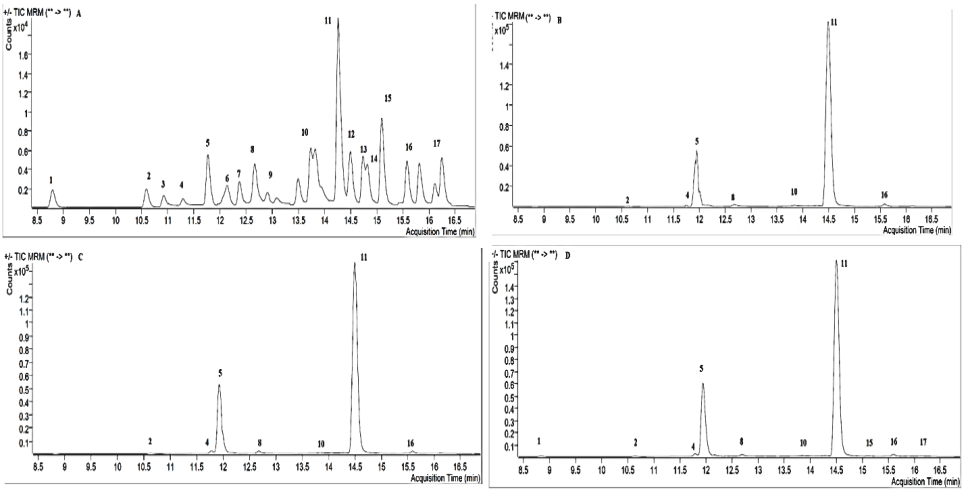

Figure 4. LC-MS/MS total ion chromatograms of phenolic compounds (A: standards, B: MDAE, C: UBAE, D: HAE).1: Gallic acid, 2: Protocatechuic acid, 3: 3,4-Dihydroxyphenylacetic acid, 4: (+)-Catechin, 5: Chlorogenic acid, 6: 4-Hydroxybenzoic acid, 7: (-)-Epicatechin, 8: Caffeic acid, 9: Vanillic acid, 10: p-Coumaric acid, 11: Hesperidin, 12: Rosmarinic acid, 13: Apigenin 7-glucoside, 14: Pinoresinol, 15: Eriodictyol, 16: Quercetin, 17: Kaempferol

Determination of volatile organic molecules by GC-MS

Volatile molecules in the extract were qualitatively analyzed in electron ionization (EI) mode with Agilent Technology 7890A Gas Chromatography (GC) Mass spectrometer (MS). Chromatographic column Agilent HP-5 MS, capillary column (30 m x 0.25 mm, the film thickness of 0.25 mm). The furnace temperature was started at 40°C, followed by standing for 5 min, then at 5°C min^{-1} at 280°C and held for 5 min. Helium gas (99.999%) was used as the carrier gas. The constant flow rate is 1.5 mL min^{-1} and the injector temperature is 250°C. The extract was injected in splitless mode with 1 mL. Interpretation of the mass spectrum was performed according to the National Institute of Standards and Technology (NIST) database. GC-MS total ion chromatograms of volatile organic molecules of the samples were shown in **Figure 5**.

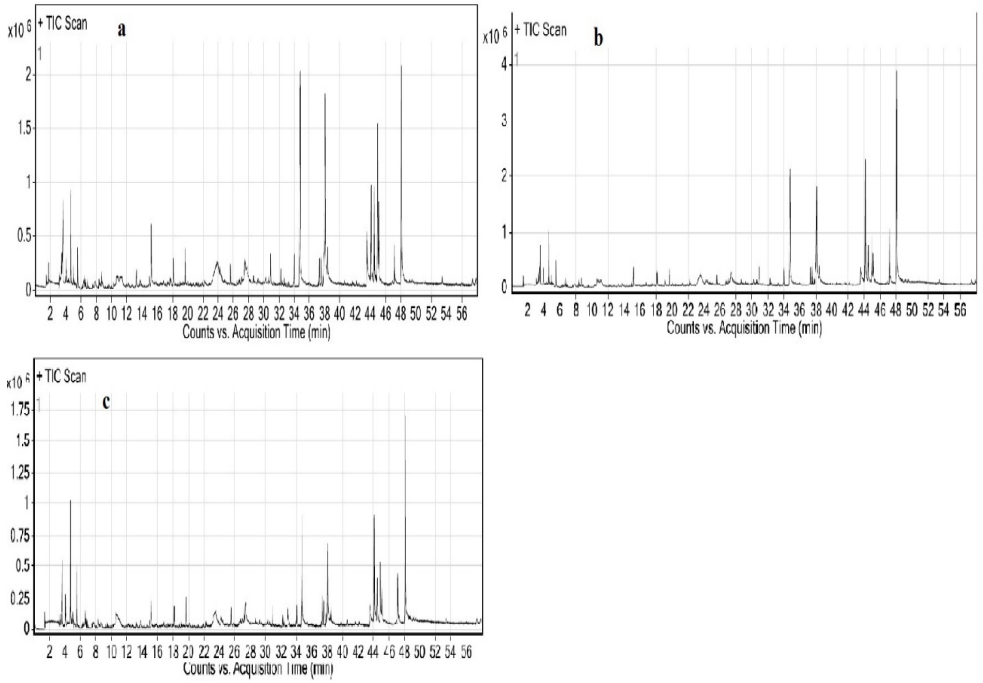

Figure 5. GC-MS total ion chromatograms and acquisition time of volatile organic compounds (a: MDAE, b: UBAE, c: HAE).

Determination of amino acid contents by HPLC-DAD

Derivatisation of samples and amino acid standards

Before HPLC-DAD analysis, amino acid (AA) standards and samples were derivatized using o-phthaldehyde (OPA) for primary AA and 9-fluorenylmethyl chloroformate (FMOC) for secondary AA according to the method of Henderson et al. (2000), modified to optimize the parameters for moringa plant leaves extraction analysis [48]. The derivatization solution was freshly prepared every day as follows: Borate Buffer: 0.4 M in water (pH 9.2), FMOC reagent, 0.2 mg/mL in acetonitrile, OPA reagent, 5 mg dissolved in 0.05 mL of methanol was added 0.45 mL of 0.4 M boric acid buffer (pH=9.5). Then 25 µL of β-mercaptoethanol was added. Derivatization of amino acids and samples was achieved by preparing a mixture of boric acid buffer/OPA/ amino acid or sample/FMOC (5v/v/v/v). The mixture was vortexed for 2 min.

HPLC-DAD analysis

HPLC-DAD analysis was performed according to Wang et al. (2010) with some minor changes [49]. Agilent 1200 Infinity series HPLC system (Agilent Technologies, CA, USA) was used for the determination of amino acids. The separation was completed on a Zorbax Eclipse Inertsil ODS-3 column (250x4.6 mm, 5 µm, Agilent). The temperature of the column oven was set at 40 °C. The mobile phase consisted of methanol /acetonitrile/ water (45/45/10, A) and phosphate buffer (pH 7.5, B). Elution was performed with the following gradient: 0–1.9 min, 100% A; 1.9-18.1 min, 0-58% B; 18.1–18.6 min, 58% B; 18.6–22.3 min, 58-70% B; 22.3–22.4 min, 70-100% B; 22.4–22.6 min, 100% B and 22.6–24 min, 100-0% B. The flow rate was 2.0 ml/min. The DAD was set at 338 nm to monitor the derivatized amino acids. The injection volume was 20 µL. Except for L theanine amino acid, 13 amino acids could be separated simultaneously with HPLC-DAD. Since the retention times of L- theanine, and tyrosine are the same, a separate chromatogram was created for L-theanine. A standard addition procedure was applied for each amino acid and validation was performed. Chromatograms of amino acids and samples were shown in **Figures 6**, and **7**.

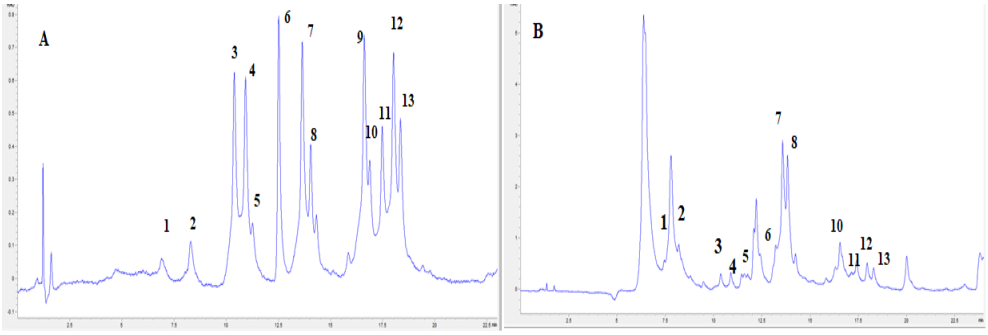

Figure 6 A: HPLC-DAD chromatogram of amino acid standards, B: HPLC-DAD chromatogram of sample (microwave digestion assisted extract)**.** Aspartic acid, 2: glutamic acid, 3: asparagine, 4: serine, 5: glutamine, 6: arginine, 7: alanine, 8: tyrosine, 9: valine, 10: tryptophan, 11: phenylalanine, 12: isoleucine, 13: leucine

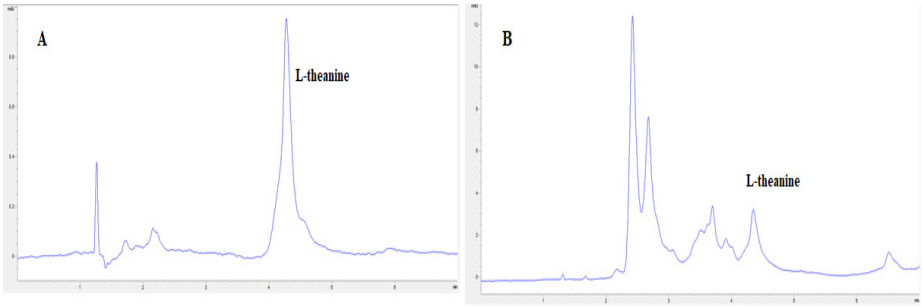

Figure 7. A: HPLC-DAD chromatogram of the standard of L-theanine amino acid, B: HPLC-DAD chromatogram of L-theanine of the sample (microwave digestion assisted extract)

Scanning Electron Microscope – Energy Dispersive X-ray Spectroscopy (SEM-EDX) Analysis

One ml of freshly prepared extracts was dropped onto the carbon band in the sample holder and dried overnight at room temperature. Dried extract samples were coated with an Au-Pd coating device (Leica). Coated samples were viewed on an SEM-GEMİNİ 500 device. Also, during the analysis, the amount of metal on the surface was determined as a percentage with the EDX detector (Energy Dispersive X-ray Spectroscopy. The metal deposits were visible in the SEM images, and we verified them with the EDX analysis. Working distance (WD) was studied in the range of 2-16 mm, and Electron High Tension (EHT) in the range of 2-5 kw. SE modes were used as a signal. Working conditions were indicated under each SEM image. EDX measurements were taken at a life time of 20 s with a voltage of 20 kV, mapping all elements detectable. For each experimental condition, three EDX measurements were taken.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) using GraphPad Prism 8.4.2. Means were separated from each other by Bonferroni's multiple comparison tests. All analyses were performed in triplicate.

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REFERENCES

1. G.E. El-Ghazali; K.S. Al-Khalifa; G.A. Saleem; E.M. Abdallah; *J. Med. Plants Res.*, **2010**, *4*, 2680–2683.
2. R. Ullah; A.S. Alqahtani; O.M.A. Noman; A.M. Alqahtani; S. Ibenmoussa ; M. Bourhia, *Saudi J. Biol. Sci*., **2020**, *27*, 2706.
3. S. Sasidharan; Y. Chen; D. Saravanan; K.M. Sundram;L. Yoga Latha; *African J. Tradit. Complement. Altern. Med.,* **2011**, *8*, 1–10.
4. R.M. Smith; *J. Chromatogr. A*., **2003**, *1000*, 3–27.
5. M.C. Hennion; V. Pichon; *Environ. Sci. Technol*., **1994**, 28.
6. S.K. Poole; T.A. Dean; J.W. Oudsema; C.F. Poole; *Anal. Chim. Acta*., **1990**, *236*, 3–42.
7. F. Chemat; Zill-E-Huma; M.K. Khan; *Ultrason. Sonochem*.,**2011**, *18*, 813–835.
8. J. Azmir; I.S.M. Zaidul; M.M. Rahman; K.M. Sharif; A. Mohamed; F. Sahena; M.H.A. Jahurul; K. Ghafoor; N.A.N. Norulaini; A.K.M. Omar; J*. Food Eng*., **2013**, *117*, 426–436.
9. http://www.epa.gov/greenchemistry/, Pubs/about_gc.html, n.d. Basics of Green Chemistry | US EPA [WWW Document]. URL, https://www.epa.gov/ greenchemistry/basics-green-chemistry (accessed 10.18.21).
10. C. Wen; J. Zhang; H. Zhang;C.S. Dzah; M. Zandile; Y. Duan; H. Ma; X. Luo; *Ultrason. Sonochem*., **2018**, *48*, 538–549.
11. F. Anwar; S. Latif; M. Ashraf; A.H. Gilani; *Phytother. Res*.,**2007**, *21*, 17–25.
12. A. Leone; A. Spada; A. Battezzati; A. Schiraldi; J. Aristil; S. Bertoli; *Int. J. Mol. Sci.,* **2016**, *17*, 2141.
13. J. Sabaté; *Am. J. Clin. Nutr*.,**2003**, *78*, 502-507.
14. Y. Cai; L. Wu; X. Lin; X. Hu; L. Wang; *Ind. Crops Prod*., **2020**, 154.
15. M.I. Nassar; El-S.A. Aboutabl; D.M. Eskander; M.H. Grace; E.D.A. El.-Khrisy; A.A. Sleem; *Pharmacognosy Res*., **2013**, *5*, 17–21.
16. X. Yang; Q. Wang; ZR. Pang; MR. Pan; W. Zhang; *Pharm. Biol*., **2017**, *55 (1),* 1207–1214.
17. V.P. Singh; A. Arulanantham; V. Parisipogula; S. Arulanantham; A. Biswas; *Eur. J. Nutr. Food Saf.*, **2018**, *8*, 204–214.
18. J.N. Kasolo; G.S. Bimenya; L. Ojok; J. Ochieng; J.W. Ogwal-Okeng; *J. Med. Plants Res.*, **2010**, *4,* 753–757.
19. S.Sreelatha; P.R. Padma; *Plant Foods Hum. Nutr*., **2009**, *64*, 303–311.
20. B. Vongsak; W. Gritsanapan; Y. Wongkrajang; I. Jantan; *Nat. Prod.Commun*., **2013**, *8(11)*, 1559–1561.
21. N. Tlili; H. Mejri; Y. Yahia; E. Saadaoui; S. Rejeb; A. Khaldi; N. Nasri; *Food Chem.*, **2014**, *160*, 98–103.
22. C. Castro-López; J.M. Ventura-Sobrevilla; M.D. González-Hernández; R. Rojas; J.A. Ascacio-Valdés; C.N. Aguilar; G.C.G. Martínez-Ávila; *Food Chem*., **2017**, *237*, 1139–1148.
23. B. Kaufmann; P. Christen; *Phytochem. Anal*., **2002,** *13,* 105–113.
24. L. Pollini; C. Tringaniello; F. Ianni; F. Blasi; J. Manes; L. Cossignani; *Antioxidants*, **2020**, *9*, 277.

25. L. Wu; L. Li; S. Chen; L. Wang; X. Lin; *Sep. Purif. Technol*., **2020**, *247*, 117014.
26. E.N. Fombang; P. Nobossé; C.M.F. Mbofung; D. Singh; *J. Food Process. Preserv.*, **2020**, *44*, 1–13.
27. B. Vongsak; S. Mangmool; W. Gritsanapan; *Planta Med*., **2015**, *81*, 1084–1089.
28. S.E. Atawodi; J.C. Atawodi; G.A. Idakwo; B. Pfundstein; R. Haubner; G. Wurtele; H. Bartsch; R.W. Owen; *J. Med. Food*, **2010**, *13*, 710–716.
29. M. Mbikay; F. Sirois; S. Simoes; J. Mayne; M. Chrétien; *FEBS Open Bio 4*., **2014**, 755–762.
30. N. Bhalla; N. Ingle; S.V. Patri; D. Haranath; *Saudi J. Biol. Sci.,* **2021**, *28*, 6915–6928.
31. D.P. Xu; Y. Li; X. Meng; T. Zhou; Y. Zhou; J. Zheng; J.J. Zhang; H. Bin Li; *Int. J. Mol. Sci.,* **2017,** *18(1)*, 96.
32. S.Saucedo-Pompa; J.A. Torres-Castillo; C. Castro-López; R. Rojas;, E.J. Sánchez-Alejo; M. Ngangyo-Heya; G.C.G. Martínez-Ávila; *Food Res. Int*., **2018**, *111*, 438–450.
33. S.O. Aisida; N. Madubuonu; M.H. Alnasir; I. Ahmad; S. Botha; M. Maaza; F.I. Ezema; S.O. Aisida; N. Madubuonu; M.H. Alnasir; I. Ahmad; S. Botha;M. Maaza; F.I. Ezema*; Appl. Nanosci.*, **2020**, *10*, 305–315.
34. B.K. Paikra; H.K.J. Dhongade; B. Gidwani; *J. Pharmacopuncture*, **2017**, *20*, 194–200.
35. J.L. Rockwood; B.G. Anderson; D.A. Casamatta; *Int. J. Phytothear. Res*., **2013**, *3*, 2278 – 5701.
36. C. Rodríguez-Pérez; R. Quirantes-Piné; A. Fernández-Gutiérrez; A. Segura-Carretero; *Ind. Crops Prod*.,**2015**, *66*, 246–254.
37. T. A. Aderinola; T.N. Fagbemi; V.N. Enujiugha; A.M. Alashi;R.E. Aluko; *Heliyon*, **2018**, 4(10).
38. S.J. Granella; T.R. Bechlin; D. Christ; S.R.M. Coelho; C.H. Paz; *South African J. Bot*., **2021**,*137*, 110–116.
39. B. Su; X. Chen; *Front. Vet. Sci*.,**2020**, *7*, 53.
40. C. Trigo; M.L.Castelló; M.D. Ortolá; F.J. García-Mares; M.D. Soriano; *Foods*, **2021**, *10*, 311031
41. N. Flórez; E. Conde; H. Domínguez; *J. Chem. Technol. Biotechnol*., **2015,** *90,* 590–607.
42. S.B. Awad; *Ultrasound Technol Food Bioprocessing*, **2010**, 545–558.
43. C.G. Stephanis; J.G. Hatiris; D.E. Mourmouras; *Ultrason. Sonochem*., **1997**, *4*, 269–271.
44. I.F.F. Benzie; J.J. Strain; *Anal. Biochem*., **1996**, *239*, 70–76.
45. W. Brand-Williams; M.E. Cuvelier; C. Berset; *LWT - Food Sci. Technol*., **1995**, *28*, 25–30.
46. M.B. Arnao; A. Cano; M. Acosta; *Food Chem*., **2001**, *73,* 239–244.
47. A.C. Gören; S. Çikrikçi; M. Çergel; G. Bilsel; *Food Chem*., **2009**, *113*, 1239–1242.
48. J.W. Henderson; R.D. Ricker; B. a. Bidlingmeyer; C. Woodward, ; *Amino Acids*., **2000**, 1–10.
49. L. Wang; R. Xu; B. Hu ; W. Li; Y. Sun; Y. Tu; X. Zeng; *Food Chem*., **2010**, *123*, 1259–1266.