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MICROWAVE-ASSISTED EXTRACTION OF FLAVONOID ANTIOXIDANTS FROM LEAVES OF *HABERLEA RHODOPENSIS*

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Abstract

Haberlea rhodopensis (Gesneriaceae) is a less explored endemic plant within a very small group of poikilohydric angiosperms that are able to survive in an almost completely dehydrated state. The purpose of the present study was to obtain extracts from Haberlea rhodopensis leaves using alternative technique- microwave-assisted extraction and to determine the total phenolic content and antioxidant activity of the extract by applying reliable methods: reducing power and ABTS radical cation decolorization assay. Different extraction conditions- microwave power and extraction times were investigated. Total phenolic content was determined using a spectrophotometric technique based on the Folin-Ciocalteu reagent and calculated as mgGAE/gDW (leaf dry weight). The results from the total phenolic assay and the antioxidant activity tests were significantly correlated. Major phenolic acids and some flavonoid- aglycones and - glycosides were analyzed by HPLC. Luteolin (3307.08 µg/gDW), myricetin (702.0 µg/gDW), ferulic acid (652.92 µg/gDW) and hesperidin (607.32 µg/gDW) were predominant in the H. rhodopensis microwave-assisted extracts and some of the effects of the plant could be attributed to the presence of these valuable constituents.

Key words: *Haberlea rhodopensis, microwave extraction, antioxidant activity, HPLC*

1.INTRODUCTION

The *Haberlea rhodopensis* belongs to the family and it is a Balkan endemic relict that is widely distributed mainly in the Rodope Mountains and some regions of the Sredna gora Mountains and the Balkan. *Gesneriaceae* is a large family distributed mainly in tropical and subtropical regions of eastern Asia, Indonesia and South America. In Europe it is represented by five species in three genera. *Haberlea rhodopensis* belongs to the so-called "resurrection plants" due to its ability to fall in anabiosis for a long time when there are unfavourable conditions and to restore back to normal when the conditions become appropriate. *Haberlea rhodopensis* is a relatively less explored plant but there is some information on its antioxidant capacity. By investigation of its phytochemical content, considerable amounts of chlorophyll and some enzymes (superoxide dismutase, citrate dehydrogenase etc.) were found and high SOD activity was reported [Yahubyan et al., 2005; Ivanov et al., 2009]. Popov et al. [2008] established that *H. rhodopensis* extracts in 1.0 and 4.0 µl/ml concentrations had radioprotective potential. A successful, simple and uniform protocol for in vitro propagation for the plant has been developed [Petrova et al., 2010]. Flavonoids, flavonoid tannins, β-carotene, ascorbate, glutathione etc. were found in other species from the *Gesneriaceae* family [Klishev et al., 1978].

The increasing interest in plant bioactive components is accompanied by a need to expand and modify the arsenal of plant-extraction protocols. Conventional techniques as heating, boiling, or refluxing can be used to extract flavonoids, however, the disadvantages are the loss of flavonoids due to ionization, hydrolysis and oxidation during extraction as well as the long extraction time (Li et al., 2005; Huang et al., 2009). Other techniques which include supercritical carbondioxide extraction, subcritical water extraction, ultrasonic assisted extraction (UAE) and microwaveassisted extraction (MAE) have also become of interest as alternatives for the conventional methods. Among these, MAE is the simplest and the most economical technique for extraction of many plant derived compounds (Zhang et al., 2005; Hemwimon et al., 2007). Extraction using microwaves can result in a yield increase in shorter time at the same temperature using less solvent. The enhancement of product recovery by microwave is generally attributed to its heating effect, which occurs due to the dipole rotation of the solvent in the microwave field.

MAE is a process of using microwave energy to heat solvents and specific molecules in contact with a sample to enhance the extraction of soluble components contained therein.Its main advantages are reduced solvent volume and time consumption, and increased sample throughput [Eskilsson et al., 2000; Zohar et al., 2005].

The purpose of this study is to research a novel, ecofriendly, rapid MAE method for the efficient large scale extraction and to evaluate the influence of the proposed extraction technique on antioxidant potential of the extracts obtained.

2. MATERIALS AND METHODS

2.1.Materials

Haberlea rhodopensis was collected from its natural habitat near Asenovgrad, Plovdiv region, Bulgaria. The plant leaves were air-dried at room temperature (25-28ºC). The dried plants were stored in air-tight dark containers until needed and roughly ground.

2.2.Chemicals

6-Hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's phenol reagent, gallic acid and 2,2-azino-bis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. All the chemicals used including the solvents were of analytical grade.

*2.3. Microwaves-assisted extraction (MA*E**)**

MAE experiments were performed with a labor microwave oven. The dried and powdered plant material (0.5 g, leaves, 1:60) was extracted with 70% v/v ethanol. The extraction was carried out under different extraction conditions: two different microwave powers- 400 and 800W and different extraction time (from 10 up to 120 sec). The residue was washed away with 70% ethanol and then the volume was adjusted to 30 ml with the solvent used for the extraction.

2.4. Determination of total phenolic content

The total phenolic content was estimated by a spectrophotometric method using Folin-Ciocalteu's phenol reagent. The total soluble phenolic compounds in the different Haberlea rhodopensis extracts were determined using Folin-Ciocalteu's reagent [Kujala et al., 2000]. 5 ml of Folin-Ciocalteu and 4 ml of sodium carbonate (7 % w/v) were added to each sample solution (1.0 ml) and standard (gallic acid). The mixture was shaken and allowed to stand for 30 min in the dark at room temperature, then absorbance was measured at 765 nm using an Anthelie spectrophotometer (Secoman, France). The

phenolic content in the extracts was expressed in terms of gallic acid equivalent (mgGAE)/g dry weight of leaves (DW). All measurements were repeated three times.

2.5.HPLC Analysis

An HPLC analysis for phenolic acids was performed on WATER system with UV-VIS detector DuoWater 2487, Discovery C_{18} 250mm column. The detection wavelengths were 280 and 320nm. The mobile phase which consisted 2%CH_3COOH (A) and 0.5%CH_3COOH- CH_3CN (1:1) (B) was used for gradient elution. The HPLC flow rate was adjusted to 0.8 ml/min (A- 0.76ml/min; B-0.04ml/min).

An HPLC analysis for flavonoids was performed on WATER system with UV-VIS detector DuoWater 2487, Discovery C_{18} 250mm column. The detection wavelengths were 308 and 380nm. The mobile phase which consisted 2%CH_3COOH (A) and CH_3OH (B) was used for gradient elution. The HPLC flow rate was adjusted to 1.0 ml/min (A- 0.7ml/min; B- 0.3ml/min).

An HPLC analysis for flavonoid glycosides were performed on WATER system with UV-VIS detector DuoWater 2487, Discovery C_{18} 250mm column. The detection wavelength was 370nm. The mobile phase which consisted 2%CH_3COOH (A) and CH_3CN (B) was used for gradient elution. The HPLC flow rate was adjusted to 1.0 ml/min (A- 0.8ml/min; B- 0.2ml/min). The analysis was performed at 26ºC.

Phenolic, flavonoid-aglycon and -glycosid compounds of each sample were identified by comparing their relative retention times with dose of the standards mixture chromatogram. The concentration of an individual compound was calculated based on peak area measurements and then converted to mg compound/g dry weight (DW).

2.6. Antioxidant Assays

The antioxidant activity of the plant extracts was determined by *in vitro* methods such as the ABTS radical scavenging activity and reducing power methods. All the assays were carried out in triplicate using an Anthelie spectrophotometer (Secoman, France) and the average values were considered.

2.6.1. ABTS radical cation decolorization assay

The total antioxidant activity of the samples was measured by improved ABTS radical cation decolorization assay according to the method of Re et al. [1999]. The unit of total antioxidant activity is defined as the concentration of Trolox having equivalent antioxidant activity expressed as mMTE/g DW. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. Afterwards, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7±0.02 at 734nm and equilibrated at 30ºC. After addition of 1.0ml of diluted $ABTS^{\bullet+}$ solution to 10µl of samples, the absorbance reading was taken at 30ºC after 6 min. Following the absorbance readings, the percentage of inhibition of $ABTS^{\bullet+}$ from the obtained extracts was calculated for each sample using the following formula: %Inhibition=$[(A_B-A_E)/A_B]$x100, where A_B=absorbance of the blank sample, and A_E=absorbance of the plant extract.

In order to determine Trolox equivalent antioxidant capacity (TEAC), the results were expressed as mM Trolox equivalents (TE) per gram dry leaf weight (DW).

2.6.2. Reducing power

The reducing power of the extracts was determined by the method of Oyaizu [Oyaizu, M. 1986]. Different concentrations of the extract (1ml) were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1%). The mixture was incubated at 50ºC for 20 min.

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Aliquots (2.5ml) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10min at 3000rpm. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. The increased absorbance of the reaction mixture indicated the increased reducing power. Vitamin C was used as positive control.

2.7. Statistical analysis

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Correlations among data obtained were calculated using Pearson's correlation coefficient (r).

3.RESULTS AND DISCUSSION

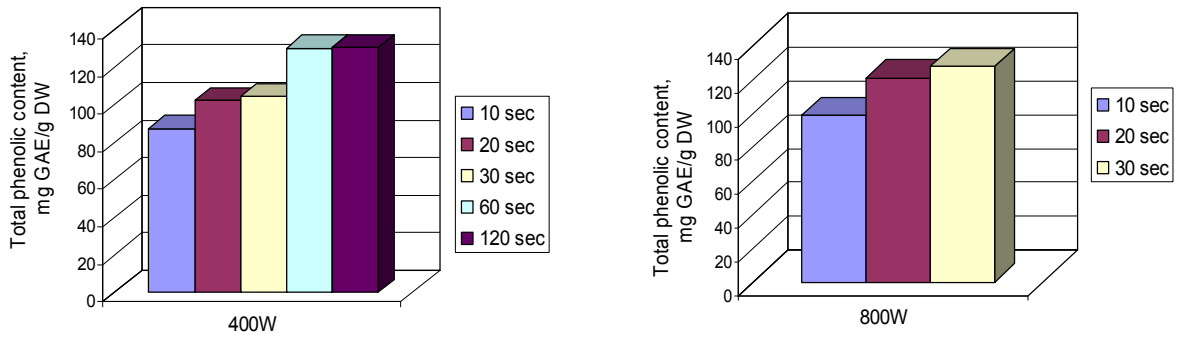
3.1. Total phenolic content

In the present work, microwave- assisted extracts obtained with 70% ethanol under different conditions (extraction time and microwave power) from *Haberlea rhodopensis* were evaluated for their total phenolic content. Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of the plant may contribute directly to its antioxidant action. The phenolic concentration in the examined plant extracts estimated using Folin-Ciocalteu`s reagent was expressed in terms of gallic acid equivalent per gram of dry plant leaf weight (DW) (Figure 1. A, B). The standard curve equation y=0.0092x+0.0367, R^2=0.9986 was used. The phenolic concentration in the examined extracts ranged from 87.39 to 130.83 mgGAE/gDW by 400W and from 98.46 to 120.68 mgGAE/gDW by 800W. Therefore, the phenolic content in the plant extracts of *Haberlea rhodopensis* leaves depends on the investigated parameters- extraction time and microwave power used for the extraction. The experimental results demonstrates that the phenolic content by 400W increase with the increasing of the extraction time from 10 to 60 sec and do not change significant from 60 to 120 sec. The importance of the extraction time can be presumed to be related to the time required for the desorption process to take place (Chen et al., 2008). Therefore, 60 sec was considered as the appropriate extraction time for extraction at 400W. The phenolic content at 800W is higher in comparison with the same time for extraction at 400W.

Microwave irradiation energy disrupts hydrogen bonds, because of microwave-induced dipole rotation of molecules and migration of dissolved ions. Microwave irradiation energy can enhance the penetration of the solvent into the matrix and deliver efficiently to materials through molecular interaction with the electromagneticfield and offer a rapid transfer of energy to the solvent and matrix, al lowing the dissolution of components to be extracted (Duan et al., 2001; Hu, 2003; Guo et al., 2003).

The analysis of total polyphenols of different extracts obtained suggested that the microwave-assisted method was effective extraction method and it could be used as method to extract antioxidant components considering factors such as the extraction time and the solvent wastage.

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A B

Figure 1. Total phenolic content of microwave extract from *H. rhodopensis* leaves, mgGAE/gDW

A- 400W

B- 800W

3.2. Phenolic acids, flavonoid- aglycones and –glycosides content

Table 1. Phenolic acids content of microwave (400W) extract from *H. rhodopensis* leaves

Phenolic acids	Content, µg/ml extract	Content, µg/g DW
Gallic acid	1.018	61.08
3,4-diOH Benzoic	0.638	38.28
2 - OH Benzoic acid	2.016	120.96
Chlorogenic acid	1.138	68.28
Vanillic acid	4.125	247.5
Caffeic acid	7.373	442.38
Syringic acid	1.516	90.96
p-Coumaric acid	5.774	346.44
Sinapic acid	6.892	413.52
Ferulic acid	10.882	652.92
Cinnamic acid	0.827	49.62

Table 2. Flavonoid- aglycones and -glycosides content of microwave (400W) extract from *H. rhodopensis* leaves

Flavonoid-aglycones and -glycosides	Content, µg/ml xtract	Content, µg/g DW
Myricetin	11.7	702
Hesperidin	10.122	607.32
Quercetin	3.135	188.1
Luteolin	55.118	3307.08
Kaempferol	-	-
Apigenin	-	-
Rutin	6.023	361.38
Hyperosid	9.642	518.52

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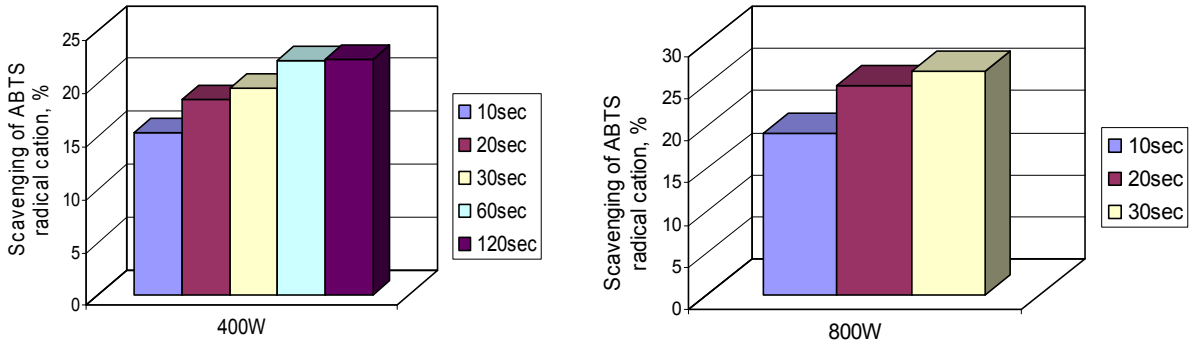
Phenolics or polyphenolics are secondary plant metabolites that are ubiquitously present in plants and plant products. They are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [Okudu et al., 1994; Bahchevanska et al., 2009; Tepe et al., 2006].

In view of the represented results in Figure 1A, B. which shown no significant difference between the two investigated microwave powers it was concluded to use 400W microwave power for our further experiments.We were interested in determining the phenolic acids and flavonoids present in the extracts and estimating their quantity. Therefore, the plant extract obtained from dry and ground *Haberlea rhodopensis* leaves with 70% ethanol at 400W was subjected to HPLC analysis (Table 1.). Several phenolic compounds were identified in the sample. These were mainly phenolic acids such as 2-OH benzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, sinapic acid, ferulic acid and cinnamic acid and flavonoids such as myricetin, hesperidin, quercetin and luteolin. The predominant phenolic acids were ferulic acid (652.92 µg/gDW), caffeic acid (442.38 µg/gDW), sinapic acid (413.52 µg/g DW) followed by p-Coumaric acid. The predominant flavonoids in the investigated sample were luteolin (3307.08 µg/gDW) followed by hesperedin (607.32 µg/gDW). Luteolin was the dominant identified compound in the investigated sample. The results strongly suggest that phenolics (phenolic acids, flavonoid-aglycones and -glycosides) are important components of this plant.

3.3. *ABTS radical scavenging activity*

The ABTS assay is a method of evaluating the total antioxidant power of single compounds and complex mixtures of various plants [Miller et al., 1996]. This assay is based on the antioxidant's ability to react with $ABTS^{\bullet+}$ radical cation generated in the assay system. The results of ABTS assays are expressed as ABTS scavenging, % and TEAC, mMTE/gDW (the standard curve equation: $y=20.762x+1.6351$, $R^2=0.948$). Higher values indicate that a sample had a stronger antioxidant activity.

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A B

Figure 2. ABTS scavenging activity of microwave extracts from *H. rhodopensis* leaves, %

A- 400W

B- 800W

Table 3. Antioxidant activity of microwave extracts from *H. rhodopensis* leaves

Extracts	TEAC, mMTE/gDW
400 W	
•10 sec	•0.864
•20 sec	•1.066
•30 sec	•1.122
•60 sec	•1.298
•120 sec	•1.303
800 W	
•10 sec	•1.092
•20 sec	•1.456
•30 sec	•1.558

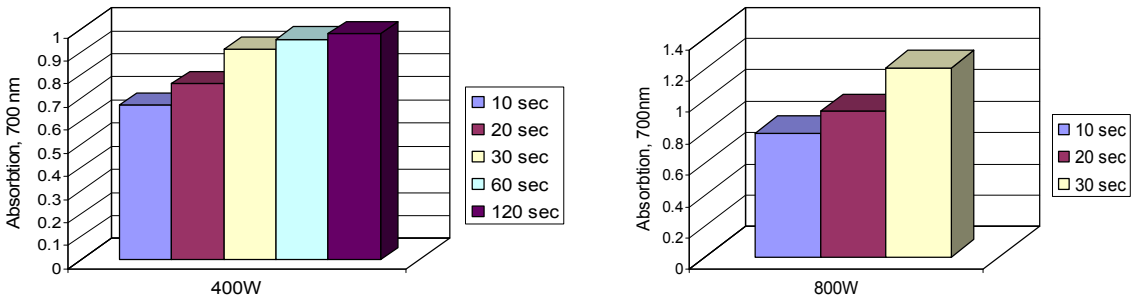
The experimental results (Figure 2. A, B) showed that the extracts (20 times diluted) obtained with 70% ethanol and for 60 sec extraction at 400W had the highest ABTS radical cation scavenging activity (22.23%). Table 3. shows the results on the TEAC of the *H. rhodopensis* extracts. It is evident that the extract which had the highest total phenolic content values (130.83 mgGAE/gDW), ranked the highest TEAC according to the ABTS assay (1.303 mMTE/gDW). This fact can be explained by the probable presence of some phytochemicals such as flavonoids and anthocyanins, known as strong scavenging and antioxidant agents, in the *H. rhodopensis* extracts. These results are in general agreement with numerous investigations of the antioxidant activity of plant extracts which have

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confirmed a high linear correlation between the values of phenolic concentration and antioxidant activity [Borneo et al., 2009; Katalinić et al., 2004].

3.4. Reducing power

The reducing ability of a compound generally depends on the presence of reductants, which exhibit antioxidant potential by breaking the free radical chain, donating a hydrogen atom. The presence of reductants (i.e. antioxidants) in *Haberlea rhodopensis* leaf extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. We investigated the reducing capacity of several extracts 20 times diluted by measuring the Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. High absorbance indicates high reducing power (Figure 3.A, B). The 70% ethanol extracts obtained for 60sec at 800W (1.203) had higher reducing power than the 70% ethanol extracts obtained at 400W for 60sec (0.9105). The reducing power of the *H. rhodopensis* 400W and 800W ethanol extracts were comparable with the vitamin C standard: 0.16 mg/ml and 0.24 mg/ml, respectively.



A B

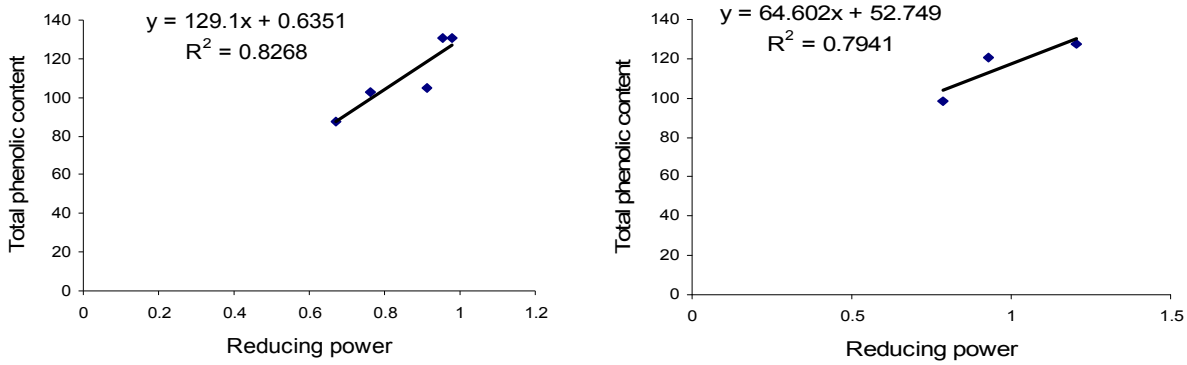
Figure 3. Reducing power of microwave extracts from *H. rhodopensis* leaves

A- 400W

B- 800W

3.5. *Correlations*

The correlation coefficient (R^2) between the total phenolic content (mgGAE/gDW) and reducing power for the microwave-assisted extracts from *H. rhodopensis* leaves was determined (Figure 4.A, B). A positive correlation was observed: R^2=0.8268 at 400W and R^2=7941 at 800W. This suggested that the major antioxidant activity of *Haberlea rhodopensis* was due to phenolic compounds and the remaining activity may be attributed to the other compounds present in the leaves. It has been confirmed for many herbs that high phenolic content is an important factor in determining antioxidant activity [Borneo et al., 2009; Kiselova et al., 2004; Javanmardi et al., 2003].



A B

Figure 4. Correlation between total phenolic content and reducing power for microwave extracts from *H. rhodopensis* leaves

A- 400W

B- 800W

4. CONCLUSIONS

In this study a new alternative method for the bioactive metabolites extraction (microwave- assisted extraction) was applied. There have been no studies on the effects of microwave irradiation on phenolic content and antioxidant capacity of *Haberlea rhodopensis*. However, some antioxidant compounds are likely to be heat labile. The antioxidant activity measured with ABTS, DPPH and Reducing power assays activity highlighted that the extracts obtained using microwaves were rich in antioxidant metabolites and can be use as a good source of antioxidants.

In order to evaluate the effect of microwave power (MAE), two different microwave powers were controlled 400 and 800 W. The represented results are shown no significant difference between the investigated microwave powers The analysis of total polyphenols of different extracts obtained suggested that the microwave-assisted method was effective extraction method and it could be used as method to extract antioxidant components considering factors such as the extraction time and the solvent wastage.

Furthermore, a significant linear relationship existed between antioxidant activity and phenolic content, indicating that phenolic compounds are major contributors to antioxidant activity. Thus, the MAE was found as suitable for obtaining of leaf extracts and the use of this alternative in analytical laboratories should increase in the next few years, especially thanks to the reasonable cost of the equipment, suggesting that this field of application should expand in the near future.

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