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Extraction Evaluation of Antioxidant Constituents of *Phillyrea Latifolia* L. (Wild Olive) from Strandja Mountain, Bulgaria

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Abstract

Phillyrea latifolia L. have played a very interesting role in the well-known historical medicine of the Mediterranean region. The wild shrub is widely distributed in Strandja mountain, Bulgaria where the ethno medicinal description of "Grypa" (local name) as an anti-inflammatory and antioxidant herb is missing. The purpose of the present study was to investigate the optimal extraction conditions by total polyphenol and flavonoid contents, rutin (quercetin-3-O-rutinoside) assay, and antioxidant capacity (AOC) of the *Phillyrea latifolia* leaves extracts. Several operational extraction parameters were examined: the extraction time, the extraction method, the extraction solvent (ethanol in water concentration), the extraction temperature, the harvest season, and the drying conditions of the raw herb. All analyses were quantified spectrophotometry. The AOC was evaluated by use of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. This study demonstrates that the ultrasound-assisted extraction (UAE) and conventional extraction at 50 °C with 50 vol. % ethanol in water solution give the best antioxidant and polyphenols yields. Likewise, examination of the leaves collected in winter shows no significant differences with those collected in summer as determined by the extracted antioxidant polyphenols. In general, the seasonality doesn't affect the antioxidant power contrary to the temperature applied (40°C) during the drying process. This study provides the opportunity to use the green parts of *Phillyrea latifolia* regardless of the harvest season as a high added value antioxidant herb.

Keywords: *Phillyrea latifolia*; Antioxidant constituents; Seasonality; Drying conditions; Ultrasound-assisted extraction; Extraction optimization

Introduction

In the last two decades interest in plant-derived food additives has grown. There are many plants grown in Bulgaria, that contain natural antioxidants and it is not investigated, yet. Members of the *Phillyrea* (Oleaceae family) are widely represented in the Mediterranean and Iberian flora, and consist of a group of plants, many of which are used in traditional medicine for many centuries. *Phillyrea latifolia* represents quite a common shrub in the Mediterranean coastal maquis. The first information about the medicinal use of the leaves of privet dated from 1st century BC where chewed against oropharyngeal inflammations, while the aerial parts were used against burns and headaches [1].

In Spain, Mediterranean Europe, North Africa, and even south of France people have used infusions and decoctions prepared from the leaves and fruits of *Phillyrea latifolia* as an astringent, diuretic [2], as antipyretic, as diaphoretic [3], as hypotensive [4] for the treatment of mouth ulcers, inflammations [5,6], aphthae [7], antispasmodic against stomach aches [8] and jaundice [9]. The flowers of *Phillyrea latifolia* were used against a migraine [6] and the fruits of it were probably in the past harvested and eaten as wild olives [10].

At the present time the use of *Phillyrea latifolia*, also in the Mediterranean region, though very restricted, seems to be still alive at least in some remote villages of the central Sardinia, Morocco and in the Bohumed district-Rif region [5,10,11]. Recent studies on the wild olive leaves indicate that it can be successfully applied against helminths [12].

Studies conducted on the content of bioactive components in wild olive leaves reveal the contents of flavonoid and flavonol glycosides (quercetin, rutin, luteoloside, apigenin, luteolin) [6,9,13,14], phenylpropanoid glycosides (salidroside, syringing, coniferin), triterpenoids (oleanolic acid, ursolic acid) [9], and lignans (phillyrin) [15]. Oleuropein and its tyrosol derivatives represent the major compounds in *Phillyrea latifolia* collected from Israel, followed by flavones (luteolin and quercetin) and their glucoside derivatives (lutolin-7-O-glucoside, apigenin-7-O-glucosin, 7-O-glucoside,) [12]. Anthocyanins such as cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside have been found in the fruits of *Phillyrea latifolia*. [16]. The volatile components of the fresh fruits of this plant have also been investigated [17].

The rich antioxidant nature of wild olive have confirmed the ethnomedicinal description of *Phillyrea latifolia* leaves as anti-inflammatory, antibacterial and antioxidant agent [18].

Although the herb has proven its useful properties over the years there is still a lack of knowledge in Bulgarian folk medicine and phyto-literature about the phytotherapeutic uses and the antioxidant capacity of *Phillyrea latifolia*. All this has been led to be carried out this investigation. Likewise, the wide distribution of *Phillyrea latifolia* in Bulgaria (Southern Black Sea Coast, Struma River Valley, southern Pyrin, Eastern Rhodopes) [19] and search for new natural sources reputed in traditional medicine led us of examine and explore its antioxidant potential.

It is known that an appropriate extraction of phenolic compounds in the plants depend on multiple factors, such as their chemical nature, raw material, storage time and conditions.

Polyphenol content, radical scavenging capacity and an extraction optimization of the *Phillyrea latifolia* leaves have rarely been studied. Thus, it is necessary to adjust the sample preparation procedure to achieve the optimal estimation of the phenolic compounds.

In this investigation, we followed to selected previous steps of the extraction optimization of *Phyllirea latifolia* leaves and to evaluate the optimal conditions performing following test: total polyphenol content (TPC), total flavonoid content (TFC), rutin assay (RA), and antioxidant capacity (AOC).

Initially, the effect of the drying conditions on collected fresh leaves was investigated by evaluating extracting solvent (ethanol in water mixtures) concentration at a chosen extraction time. Once the optimal solvent concentration was found, the extraction time was examined at constant extraction parameters (temperature, particle size, and solid-to-solvent ratio). This was also followed by studying the kinetic by total dry plant material obtained after extraction processes. The kinetics were fallowed to establish the equilibrium of the extraction process for a better understanding of the extraction process. In addition, the influence of the seasonality, the effect of the extraction method and the effect of extraction temperature for *Phyllirea latifolia* for the presence of antioxidant polyphenols were also investigated. As to our knowledge, there is no study reported regarding the parameters mentioned above for *Phillyrea latifolia*.

Materials and Methods

Plant materials

The leaves from *Phillyrea latifolia* were collected in Strandja mountain, near the Black Sea region (the area “Parnara”, near village Varvara), in the south-eastern of Bulgaria. The samples of two different seasons of harvest were examined: picked up in the summer (August, 2015), and picked up in the winter (March, 2016). The temperatures measured on the days of collection of the plant were 29 °C in August, and 7 °C in March. To ensure a representative sampling, 1 kg of the wild plant leaves were collected. The *Phillyrea latifolia* L. was identified by experienced biologists from the National Park Strandja. The leaves were kept in a dry place for the further experiments.

Chemicals

Ethanol 96 % was supplied by Valerus, (Sofia, Bulgaria), Methanol, HPLC grade; Sodium carbonate (> 99 %); Gallic acid anhydride (> 99 %); Sodium nitrite, Aluminum chloride hexahydrate - by Merck, (Sofia, Bulgaria), Folin-Ciocalteau reagent - 2N solution, 2,2-diphenyl -1-picrylhydrazyl (DPPH), Rutin hydrate, ammonium hepta molybdate tetrahydrate ($NH_4Mo_7O_{24}$ x $4H_2O$), Quercetin hydrate (≥ 95 %), Sodium hydroxide, were supplied by Sigma Aldrich, (Sofia, Bulgaria). Ammonia-iron alum - by Sharlau, (Sofia, Bulgaria). Deionized water from water deionizer -Elix70C Gulfstream, supplied by Merck (Sofia, Bulgaria).

Extraction procedure

For the experiments leaves of *Phyllirea latifolia* were gathered in the summer and winter harvest seasons. One part of the summer leaves was dried at RT (approx. 25 °C) for a week, another one was dried using a laboratory oven at 40 °C for 4 h. All samples were kept in a dry place for a year before being ground in the grinder and sieved. The leaves used contained water, not more than 10%. For the experiments, a fraction of 0.5 –1.0 mm particle size was used. The solid-to-solvent ratio was fixed at 1:10 (1 g *Phyllirea latifolia* leaves in 10 mL solvent) for all analyses. The extraction kinetics were carried out with 50 vol.% ethanol in water solution at RT through magnetic stirring at 1411 RCF

(relative centrifugal force) with a magnetic stirrer (MS-H-Pro+, Dragon Laboratory Instruments, Beijing, China) for 5, 10, 30, 60, 90 minutes, and for 5, 30, 60, 90, 120 minutes. Another extraction kinetic was performed applying ultrasound power at 25 kHz on an ultrasound bath (AU-32, ARGOLAB, Italy) at RT for 5, 30, 60, 90, 120 minutes. Likewise, the kinetic presenting the influence of the temperature on the extraction was carried out by stirring at 50 °C with 50 vol.% ethanol in water solution for 5, 30, 60, 90, 120 minutes. The influence of the solvent composition (10, 30, 50, 70, 90 vol.%) ethanol in water was studied at RT for 60 min extraction time by ultrasound-assisted extraction (UAE). The effect of the drying conditions was evaluated by UAE for 60 min with 50 vol.% ethanol in water solvent. The kinetic study on total dry mass was performed by weighing the used plant material to a constant mass. After each extraction, the plant material was carefully pressed, and the extract was filtered through cotton and filter paper, weighed and analyzed immediately after the appropriate dilution. All analyses were made in duplicate.

Total polyphenol content by the Folin–Ciocalteau Method

The total polyphenol content of *Phyllirea latifolia* leaves extract was determined according to the Folin & Ciocalteu's assay [20] applying gallic acid as a standard solution. In general, 0.3 mL of the extract was added to a 10 mL volumetric flask containing deionized water. An aliquot of 0.02 mL of the diluted solution and 0.3 mL of Folin Ciocalteu reagent was added into a test tube containing 1.58 mL of distilled water. A minute later a 0.3 mL of 200 g/L Na_2CO_3 was added. The mixture was placed in the dark at room temperature for 120 minutes and then was measured the absorbance at 765 nm wavelength using a UV-VIS spectrophotometer (T60UV/VIS, Oasis Scientific Ltd, South Carolina, USA), using a 10 mm path length cuvette.

The results were calculated as gallic acid equivalents, using a standard curve: Abs=1.016x, (R^2=0.9984) obtained with standard solutions of gallic acid (0.1 - 1.0 mg/mL). The total phenolic content of the *Phyllirea latifolia* extracts was expressed as milligrams of Gallic acid equivalents per gram dry weight (mg GAE/g dw) and calculated by the following formula:

$$TPC = C \times V \times \frac{F}{M} \quad \ldots\ldots\ldots (1)$$

where TPC is the total polyphenol content, mg GAE/g dw; C is the concentration of the gallic acid, mg/mL; V is the volume of the solvent used, L; F is the dilution coefficient of the sample; M is the mass of the sample, g.

Total flavonoid content

The formation of a flavonoid– aluminum complex was employed to determine the flavonoid content [21]. An aliquot of 1 mL previously diluted extract (0.3 mL of the extract was added to a 10 mL volumetric flask containing deionized water), 4 mL of deionized water, and 0.3 mL of 5% $NaNO_2$ was dropped into a 10 mL volumetric flask. After 5 minutes, 0.3 mL $AlCl_3$ (10%) was added and 2 mL of 1M NaOH. Finally, deionized water was added to reach a volume of 10 mL.

A standard curve of quercetin was prepared ((0.5-5.0) g/L, y= 0.2175x, R^2=0.9936) and the results are expressed as quercetin equivalents per gram dry weight (mg QE/g dw) and calculated by the following formula:

$$TFC = C \times V \times \frac{F}{M} \quad \ldots\ldots\ldots (2)$$

where TFC is the total flavonoids content, mg QE/g dw; C is the concentration of quercetin, g/L; V is the volume of the used solvent, L; F is the dilution coefficient of the sample; M is the mass of the sample, g.

Rutin assay

The rutin assay was performed according to The International Pharmacopoeia method after modifying the method [22]. An aliquot of 1 mL previously diluted extract (0.3 mL of the extract was added to a 10 mL volumetric flask containing deionized water) and 1 mL ammonium molybdate (100 g/L) was added to a 10 mL volumetric flask containing deionized water. After that, the solution was measured at 360 nm. The content (mg/g dw) of rutin (R) in the samples was calculated as follows:

$$RA = A \times Co/Ao \times C \quad \ldots\ldots\ldots\ldots (3)$$

Where RA is the rutin assay; A is the absorbance of the sample; A_o is the absorbance of the standard; C_o is the concentration of the standard solution of the rutin (g/mL); C is the concentration of the sample, (g/mL).

Antioxidant capacity by the DPPH Method

Radical scavenging activity was determined according to the technique reported by Loizzo et al. [23]. One milliliter of the extracts with different concentrations (0.05 – 1.0 mg/mL) was added to a test tube containing 4 mL of 0,004% DPPH solution. The mixture was shaken well and placed in the dark at room temperature for 60 minutes. The absorbance was measured at 517 nm.

Antioxidant activity defined as the extract concentration necessary to neutralize 50 % of free radicals - IC_{50} is calculated by plotting the correlation between the concentration of the extract (mg/mL) and inhibition (%) - C/I.

Free radical scavenging ability of the tested samples was calculated using the formula [24]:

$$IC\ (\%) = \left(Ao - \frac{Aa}{Ao}\right) x\ 100 \quad \ldots\ldots\ldots\ldots (4)$$

where IC is the inhibition capacity, %; A_o is the average value of the absorbance of the blank; A_a is the average value of the absorbance AOA; IC is the inhibition capacity;

After recalculation, the results were expressed as the IC_{50} values (μg/mL).

Total dry mass

The total dry mass of the plant material obtained after extraction was determined in accordance with the method of Ph. Eur. (European Pharmacopeia) with some modifications [25]. In flat-bottomed dishes were introduced rapidly used drug previously well pressed and weighted. The plant mass was dried at 105 °C in an oven Determ (Robotica, Velingrad, Bulgaria) for 2 hours. Thereafter, the samples were cooled in a desiccator under anhydrous silica gel and were weighted. The procedures were repeated to a constant weight. The results were calculated as gram dry mass.

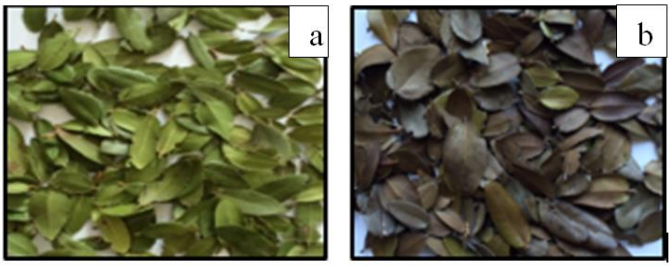
Statistical analyses

Each sample was extracted and analysed in duplicate and results are expressed as the mean ± standard deviation (SD). Statistica 9.0 software (Stat-Soft, Tulsa, OK, USA) was used for the statistical calculations.

Results and Discussion

Evaluation of the seasonality and drying conditions

There are no data available concerning antioxidant power and total polyphenol content of winter and summer leaves. Also, two different drying condition of the summer "Grypa" leaves were compared by AOC and TPC; drying at RT and drying in the laboratory oven at 40 °C (Picture 1a, 1b).



Picture 1: *Phyllirea latifolia leaves dried at room temperature (RT) (a) and at 40 °C (b).*

However, it should be noted that the collection period, as well as the soil conditions, may affect the bioactive ingredients from different plant species: for instance, salt in soil modify flavonoid composition in *Phyllirea latifolia* [26]. Furthermore, plant samples in the current study were dried at 40 °C in order to minimize negative effects on polyphenols in general.

A part of the summer leaves and all winter leaves were dried at RT. All extractions were conducted with UAE with 50 vol.% ethanol in water solution for an hour. The temperature of the ultrasound bath was kept at around 25 °C.

The results pointed out in (Figures 1 and 2), that where was a gentle difference in TPC between summer and winter leaves extracts, constituting 107.5 mg GAE/g dw and 86.5 mg GAE/g dw, respectively. Likewise, the AOC shows almost similar results between both *Phyllirea latifolia* extracts – 262.0 μg/mL in the summer leaves and 272.0 μg/mL in the winter leaves.

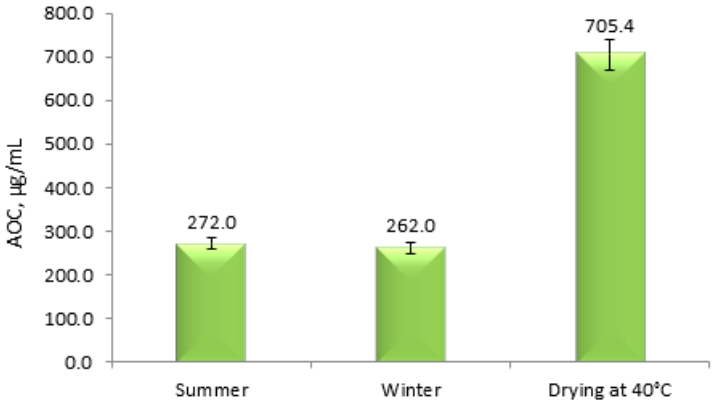


Figure 1: *Antioxidant capacity (AOC) of Phyllirea latifolia summer and winter leaves dried at room temperature (RT) and summer leaves dried at 40 °C.*

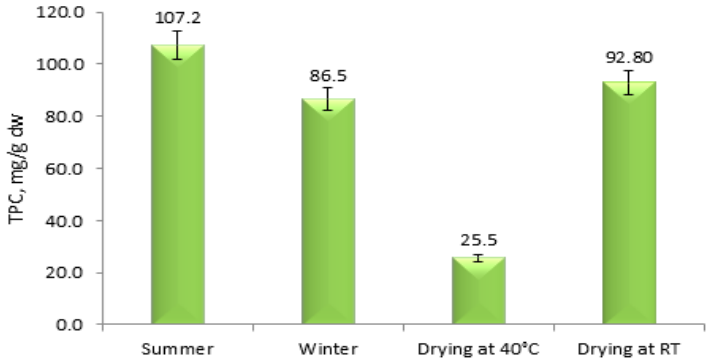


Figure 2: *TPC of Phyllirea latifolia summer and winter leaves dried at RT and summer leaves dried at 40 °C.*

The harvest season does not significantly affect the plant's antioxidant potential, according to the results obtained. The drying conditions, in contrast, indicates a significant impact in both TPC and AOC values. A major amount of TPC was obtained in *Phyllirea latifolia* leaves dried at RT - 107.2 mg GAE/g dw, which is 4.2 times higher than the value obtained from the leaves dried at 40 °C - 25.5 mg/g dw. Similarly, the AOC was higher in case of drying at RT with a value of 262.0 μg/mL, instead of 705.4 μg/mL in case of drying at 40 °C.

In general, the seasonality doesn't affect the antioxidant power contrary to the applying temperature of 40 °C during the drying process. In a commonly used protocol [27] it was reported that is not good to exceed 55 °C during drying of raw materials. In our case, the duration of the drying process at 40 °C probably is too

long or the *Phillyrea latifolia* bioactives may degrade at low temperatures.

Evaluation of the ethanol in water solvent concentration

It is known that the yield of extracted polyphenols depends on the type of solvents used and their different polarity. Ethanol is a good solvent for the extraction of polyphenols it is safe, cheap and non-toxic. Likewise, ethanolic raw extractives from *Phillyrea latifolia* leaves showed antibacterial activity [28]. Therefore, the ethanol was chosen in the present evaluation.

Several different concentrations of ethanol in water (10, 30, 50, 70, 90 vol.%) were employed to evaluate the effect of the selected solvent on the extraction antioxidant yield. The extractions were done at RT for 60 minutes extraction time by UAE. The obtained results were indicated in (Figures 3 and 4).

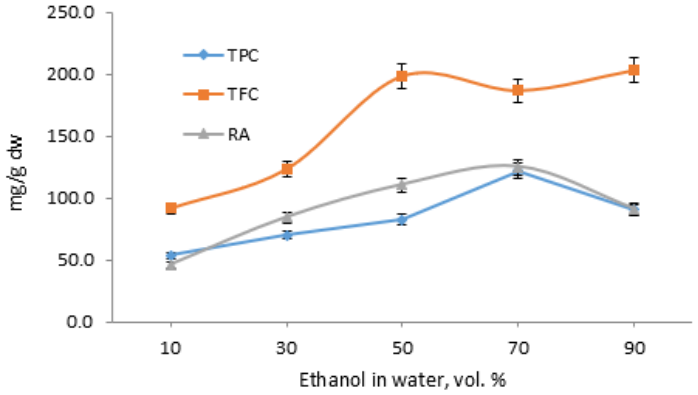


Figure 3: *The effect of ethanol in water solvents of Phyllirea latifolia leaves on the total polyphenol content (TPC), total flavonoid content (TFC), rutin assay (RA).*

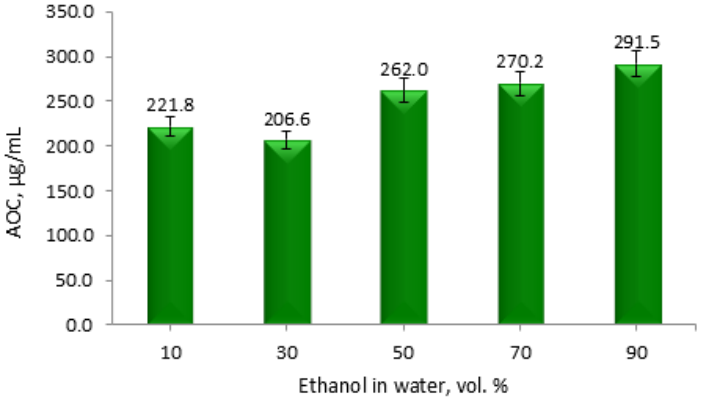


Figure 4: *The effect of ethanol in water solvents of Phyllirea latifolia leaves on the antioxidant capacity (AOC).*

The line graphs and bar chart show the TPC, TFC, RA – (Figure 3) and AOC – (Figure 4) value fluctuated in variety concentration of ethanol in water. It is apparent that the value of TFC is the highest at 50 vol.% ethanol in water concentration at the rate of 198.3 mg GAE/g dw. At the same concentration of ethanol in water, the R and TPC have 110.5 mg QE/g dw and 82.36 mg R/g dw values. However, the values of R and TPC, have the maximums at 70 vol.% ethanol in water solution – 125.0 mg R/g dw and 121.9 mg GAE/g dw, respectively.

The higher polar mixtures of ethanol in water are mean more polar extractive polyphenols in the *Phyllirea* leaves more of which are flavaonid structures-based substances, according to the obtained results.

The AOC calculated as IC_{50} (Figure 4) has a maximum value with 30 vol.% ethanol in water solution, where only 206.6 μg/mL of the extract can neutralize 50% of free radicals.

Finally, the concentrations of ethanol in water between 30 vol.% and 70 vol.% give the best yields of high-added value compounds and antioxidant power thus the 50 vol.% ethanol was chosen for optimal and used for further extraction kinetics.

Evaluation of the extracting time

In the present investigation the extraction parameter, extraction time was evaluated by TPC, TFC, RA, and AOC assays. The solid to the solvent ratio (1:10, w/v) and concertation of ethanol in water (50 vol.%) were kept constant during the extraction kinetics procedures. The evaluation of the extraction time was carried out through a sequence of extractions conducted at RT by magnetic stirring and applying US. The extraction kinetic carried out by magnetic stirring at 50 °C also was done.

The first extraction kinetic was carried out through magnetic stirring at RT and was made in the following periods of time 5, 10, 30, 60 and 90 minutes. The results obtained are presented in (Figures 5-8).

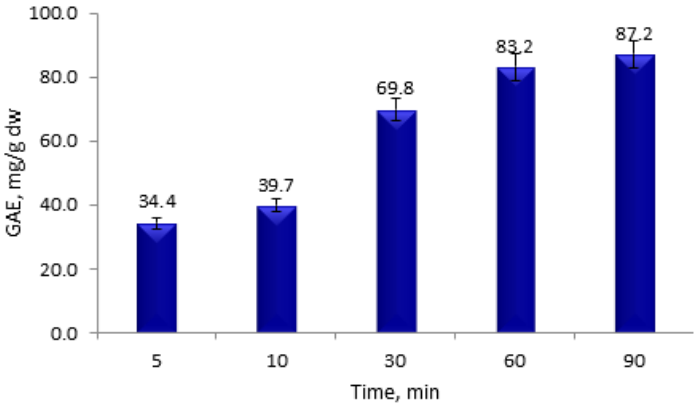


Figure 5: *Total polyphenol content (TPC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT).*

In Figure 5, it can be seen increasing from approximately 34.4 mg GAE/g dw at 5 minutes to 69.8 mg GAE/g dw at 30 minutes, the TPC of *Phyllirea latifolia* leaves remained almost stable at the stirring periods of 60 (83.2 mg GAE/g dw) and 90 minutes (87.2 mg GAE/g dw) time. For TFC, likewise, it starts with a high value of QE flavonoids, from 165.0 mg QE/g dw at 5 minutes to

210.5 mg QE/g dw at 60 minutes and only, at ratio of 2,1% is increasing at 90 minutes extracting time (Figure 6). The RA is reached to a stable value after 60 minutes with a peak of 105.3 mg R/g dw and increase with 4.5% at 90-minute (Figure 7). The same trend is observed in (Figure 8) with AOC. The stable values are observed after 60- and 90- minute extraction times where only 270.1 µg/mL and 265.5 µg/mL can reduce 50% of the free radicals.

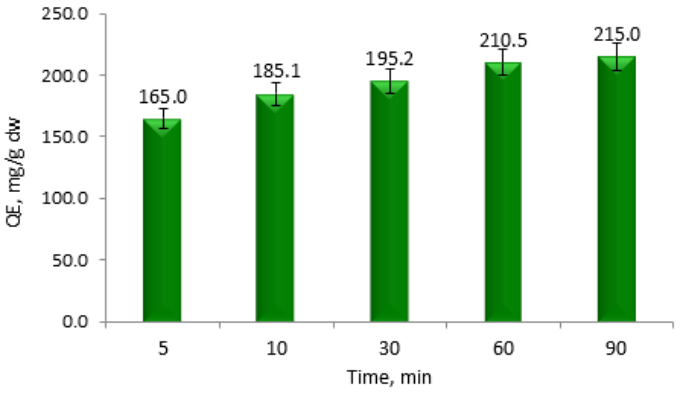


Figure 6: *Total flavonoid content (TFC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT).*

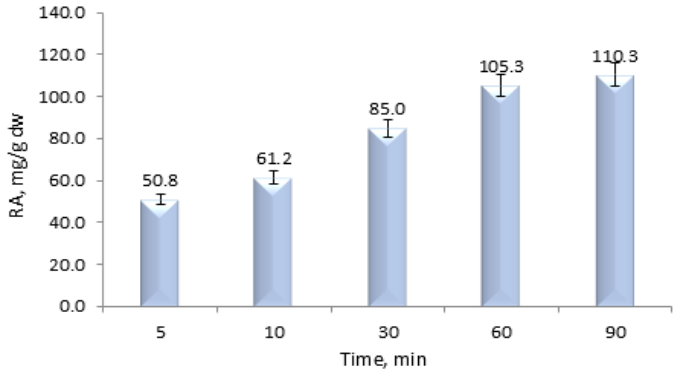


Figure 7: *Rutin assay (RA) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT).*

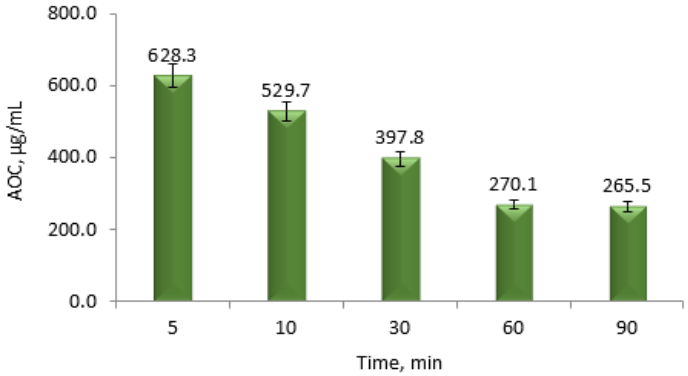


Figure 8: *Antioxidant capacity (AOC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT).*

It can be concluded that the kinetic curves reached to the limit by magnetic stirring at RT after 60 minutes extraction time. Based on the kinetic results, this time can be noted as optimal for the optimal yield of the desired components and AOC.

In the investigations of plant extracts, it is good to know the kinetics of the process also by total dry mass when equilibrium is achieved, for a better understanding of the plant material extraction. Using a gravimetric method described above, the kinetics of the total dry mass of *Phyllirea latifolia* leaves was done. and the results were plotted in (Figure 9).

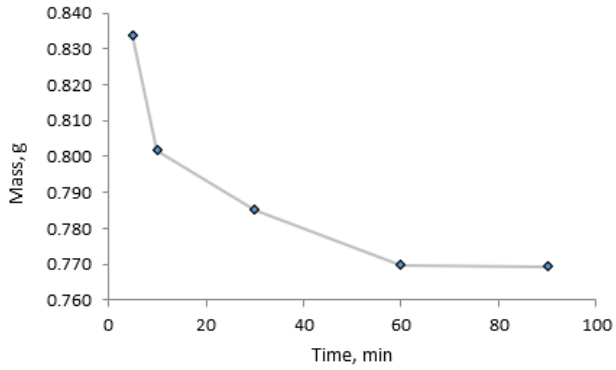


Figure 9: *Extraction kinetic by total dry mass of Phillyrea latifolia leaves extracted through magnetic stirring at room temperature (RT).*

In the kinetics presented, water content in extracted plant leaves (9.80%) is not recalculated. It is evident that the mass of the solid phase decreases steadily from 0.835 g at 5 minutes to 0.785 g at 30 minutes, then remains stable at approximately 0.770 g after stirring between 60 and 90 minutes.

The extraction efficiency may be significantly influenced by operating parameters, not only by changing the extraction time, and solvent composition but also by applying an ultrasound power and temperature [29,30].

The application of UAE to the preparation of plant samples has increased in last years. The ultrasound assistance could accelerate highly the extraction process, by increasing the yield of extracted total polyphenols and thus increasing the AOC. In the present work, the extraction kinetic by antioxidant phenolics (polyphenols and flavonoids) was carried out using an ultrasound bath. For comparison extraction efficiency between the conventional and the ultrasound-assisted methods, two extraction kinetics were carried out by magnetic stirring and by UAE. Both kinetic studies were made in the same period of times - 5, 30, 60, 90 and 120 minutes and at RT. The bar charts below (Figures 10-13) provide the comparison of extraction kinetics conducted by both extracting methods evaluated by TPC, TFC, RA and AOC assays.

Generally, extracting with the US shows much better results than stirring at the same conditions. For TPC, the highest value is recorded at 122.41 mg GAE/g dw with UAE for 120 minutes, 1.4 times greater than stirring at the same conditions - 90.3 mg

GAE/g dw (Figure 10). Similarly, the Figure 11 and Figure 12 also indicated that, in cases of TFC and RA that the 120 minutes time extraction, applying the US gives better results, which are 500.5 QE/mg dw and 140.0 mg R/g dw, respectively. The quercetin equivalent flavonoids are 2.2 times higher than those by magnetic stirring for 120 and 90 minutes.

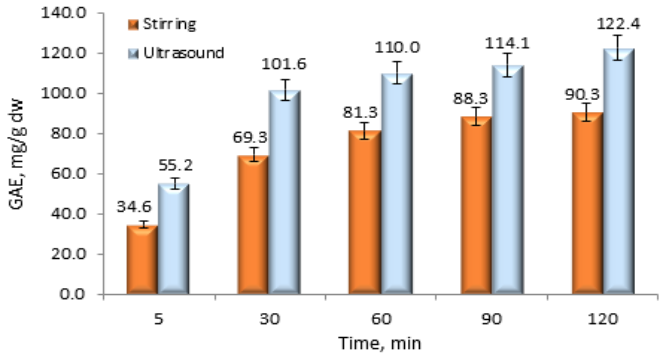


Figure 10: *Comparison of total polyphenol content (TPC) kinetics of Phyllirea latifolia leaves extracted through magnetic stirring and ultrasound-assisted extraction (UAE) at room temperature (RT).*

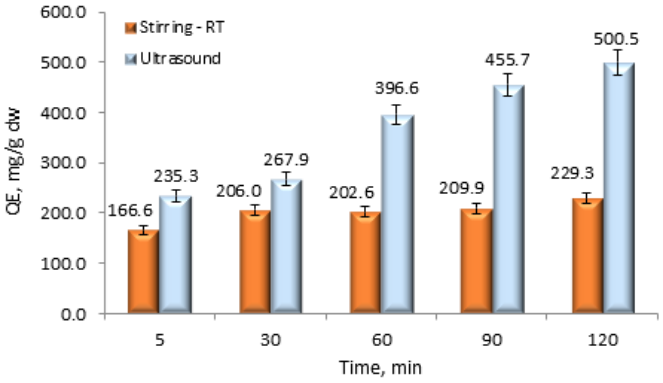


Figure 11: *Comparison of total flavonoid content (TFC) kinetics of Phyllirea latifolia leaves extracted through magnetic stirring and ultrasound-assisted extraction (UAE) at room temperature (RT).*

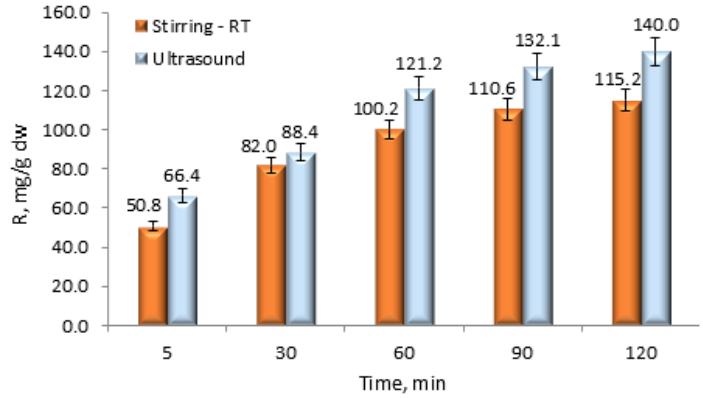


Figure 12: *Comparison of rutin assay (RA) kinetics of Phyllirea latifolia leaves extracted through magnetic stirring and ultrasound-assisted extraction (UAE) at room temperature (RT).*

The antioxidant capacity, indeed, demonstrates a result in the same trend with UAE. The most effective sample for antioxidant capacity (174.2 µg/mL), was recorded with UAE at 120 minutes, 1.7 times lower than stirring at the same time – 301.3 µg/mL (Figure 13).

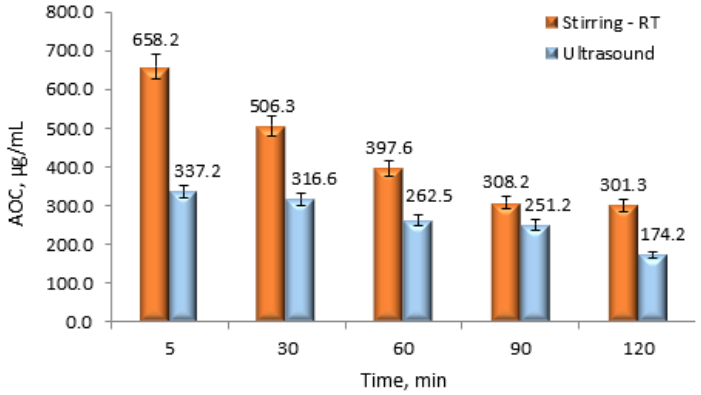


Figure 13: *Comparison of antioxidant capacity (AOC) kinetics of Phyllirea latifolia leaves extracted through magnetic stirring and ultrasound-assisted extraction (UAE) at room temperature (RT).*

Undoubtedly, the UAE at RT for 120 minutes revealed the highest results and it is more effective than magnetic stirring. The 90 minutes could be taken as optimal because of economic reasons. However, it is important to find out another extraction method or to change some of the operational parameters to decrease extraction time.

The kinetic curves by total dry mass also were made and depicted in (Figure 14).

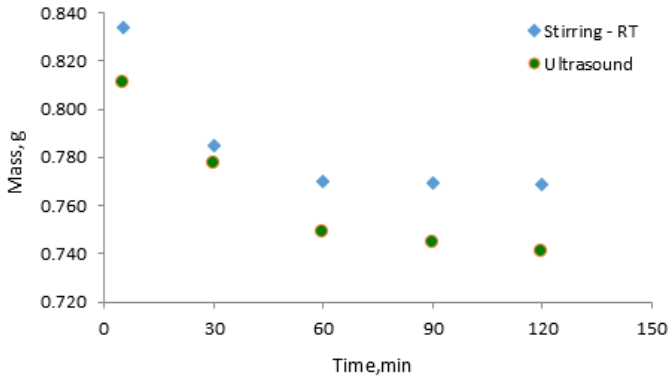


Figure 14: *The kinetic curves by the total dry mass after applying ultrasound-assisted extraction (UAE) and by magnetic stirring.*

As shown, kinetic curves have two parts with different characters. The initial steep part of the graphic corresponds to the dissolution of the readily available substances on the surface of sample particles. When applying ultrasound power, the dissolution of the substances gets faster in case of stirring. Based on the total dry mass kinetic, the plateau or the extraction equilibrium is achieved after 60 minutes for both US and stirring extractions.

The high extraction time in the kinetics above probably shows that the magnetic stirring and UAE at RT is not the best way to extract the drug of interest. There are bioactive substances which need more time for discharging, nevertheless applying ultrasonic power has a positive effect on the yields of phenolics and AOC. The UAE is an alternative to conventional extraction methods because it may improve the mass transfer, reducing the extraction time and the solvent used. However, further extraction optimization is required, with changing or modifying some of the operational extraction parameters.

Evaluation of the extraction temperature

Next step of the optimization was rising the temperature to 50 °C during extraction with a magnetic stirrer. The samples were taken at 5, 30, 60, 90, and 120 minutes. The higher temperature was applied to increase the efficiency of extractingng antioxidant polyphenols from the leaves. Results were compared with the extraction kinetics by stirring at RT from the previous kinetic study and presented in (Figures 15-18).

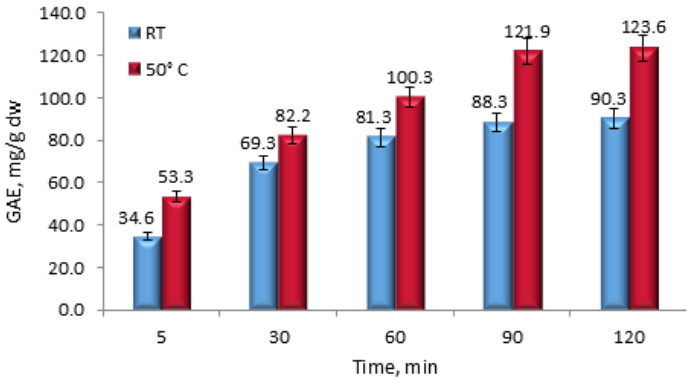


Figure 15: *Total polyphenol content (TPC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT) and 50 °C.*

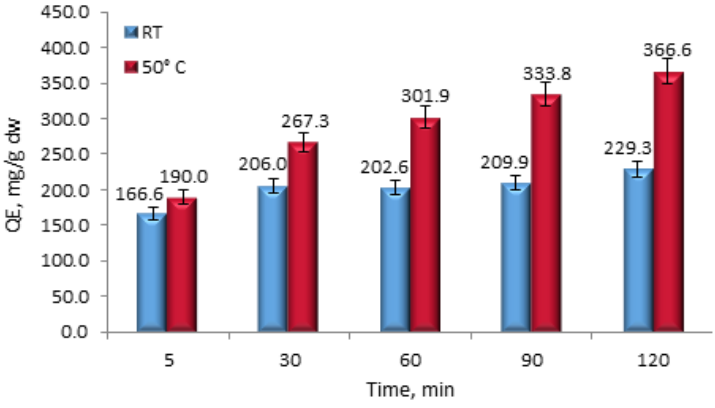


Figure 16: *Total flavonoid content (TFC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT) and 50 °C.*

The bar charts provided a comparison of TPC, TFC, RA and AOC in two different extracting temperatures: at RT and at 50 °C. For TPC, optimal time is 90 minutes with a value of 121.9 mg GAE/g dw, likewise of stirring at RT where the extracted flavonoids are increasing up over 90-minutes extraction time (Figure 15). Increasing of temperature during the extraction is even more effective than UAE at RT where the maximum of total polyphenols calculated as Galic acid is 122.4 mg GAE/g dw, which is indicated at 120-minutes extraction time.

In both TFC extraction kinetics the maximum yield is at 120-minutes (366.6 mg QE/g dw at 50 °C and 229.3 mg QE/g dw at RT) as shown in Figure 16. The stirring extraction at 50°C is 1.6 times more effective than those conducted at RT. The UAE at 120-minutes is 500.5 mg QE/g dw that is higher with 1.4 times than stirring extraction at 50 °C and 2.2 times higher than stirring extraction at RT. It can be concluded that UAE is more effective for extraction of flavonoids than stirring extraction at RT and 50 °C.

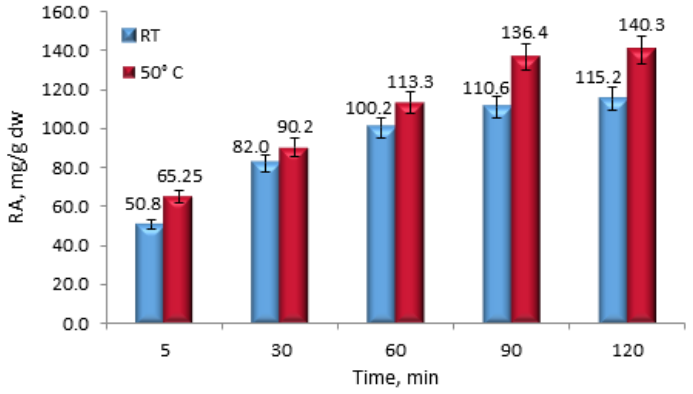


Figure 17: *Rutin assay (RA) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT) and 50 °C.*

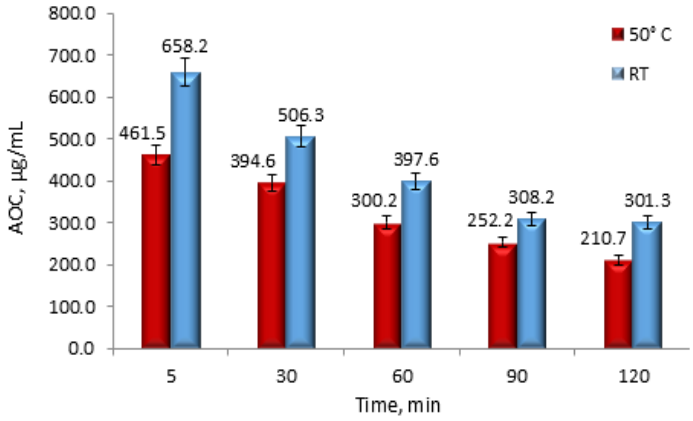


Figure 18: *Antioxidant capacity (AOC) kinetic of Phyllirea latifolia leaves extracted through magnetic stirring at room temperature (RT) and 50 °C.*

Concerning RA extraction kinetics (Figure 17) stirring extraction at 50 °C give the similar results as UAE at RT where at 120-minutes extraction time the RA is 140.3 mg R/g dw.

The value of AOC – 210.7 μg/mL is 1.2 worst then UAE extraction for 120-minutes and 1.4 times higher than RT stirring extraction values (Figure 18).

The TPC, TFC and RA values are twice at high in comparison with the values obtained by magnetic stirring at RT. The extraction at 50 °C increase the yield of the compounds of interest and obviously is effective just as much as UAE.

Conclusion

In recent decades, interest in plant sources of antioxidants has increased, which in turn has necessitated the search for new plant materials. Among plants we have selected to investigate the *Phyllirea latifolia* because of its reported pharmacological activities and its widely distribution in Bulgaria (Strandja mountain).

This study demonstrates that the UAE and conventional extraction at 50 °C with 50 vol.% ethanol in water solution exhibits the best antioxidant and polyphenols yields. Likewise, no significant differences were found between the extracted antioxidant polyphenols between leaves collected in the summer and in the winter. In general, the seasonality doesn't affect the antioxidant power of *Phyllirea latifolia* leaves. However, a temperature of 40 °C used for drying of fresh *Phillyrea* leaves influence negatively the quantities of extracted antioxidants. Our results prove that the leaves of *Phillyrea latifolia* can be used as an antioxidant drug whit health-promoting property. Our investigation is an initial step in extraction optimization of wild herb grown in Bulgaria. The present results encourage additional and more in-depth studies on the phenolic composition of the plant extracts and assessment of antioxidant activity of each compound separately. Some phenolic compounds remain to be identified. A possible phytopharmaceutical utilization of the aerial parts of this species could represent an interesting perspective for Bulgarian, particularly Strandja region.

Conflict of Interest

The authors report no conflict of interest

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