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Hyphenated Extraction of Valuable Compounds from *Aesculus carnea*: Ultrasound Extraction with Pulsed Electric Field Pretreatment

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Abstract: Wood-related procedures, such as lumberjacking and pruning, inevitably result in big piles of leaves, which are considered a major by-product. Extracting valuable compounds from natural by-products is an ongoing trend. In this work, the use of Pulsed Electric Field (PEF) was evaluated as a pretreatment step, prior to the ultrasound-assisted extraction of phenolic compounds from *Aesculus carnea* leaves. In addition, various solvent systems were examined, as well as the time of pretreatment with PEF. According to the results, up to 33% more phenolic compounds can be extracted, under optimum conditions (30% ethanol in water as solvent and PEF pretreatment for 30 min, compared to the same solvent, without PEF). Moreover, PEF treatment time was not (i.e., 30 and 60 min) and no differences were recorded, suggesting that a lower treatment time can yield the same extraction of phenolic compounds. As such, the use of PEF is highly recommended in combination with ultrasound extraction, to maximize the yield of phenolic compounds extracted from the leaves of *Aesculus carnea*.

Keywords: *Aesculus carnea*; HPLC-DAD; hydroethanolic solution; phenolic compounds; pulsed electric field; ultrasound-assisted extraction

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1. Introduction

Aesculus x *carnea* (Family: *Sapindaceae*, species: *Aesculus carnea*, Hayne) commonly known as European horse-chestnut is a large tree native to the Balkans forests of Europe. It can grow up to 40 m and it has long leaves around 15–30 cm. It is known that its leaves are a rich source of phenolics [1,2]. It was not until recently that studies have showcased that compounds found in *Aesculus* spp. exhibit even more benefits than initially believed [2]. Polyphenolic compounds derive naturally, as a result of the plant metabolism and more specifically from the acetate pathway or the shikimate pathway [1]. Polyphenolics can have a wide variety of chemical structures (and therefore, size, weight etc.), and can be composed of different monomer units [3]. As regards the monomer units of the polyphenolic compounds, there is a wide variety of monomers, resulting in an even bigger variety of polymer structures [4]. Despite the fact that polyphenolic compounds can be found free in plant tissues, usually, polyphenolic conjugates with other compounds can be found. For instance, carbohydrate sugars are one such class of compounds that usually forms conjugates with polyphenolics. Conjugation is achieved mainly via the hydroxyl group of the polyphenolic compound that usually binds to the sugar residues naturally present in the plant. Such compounds can vary in chain length and composition. However, glucose is most often conjugated with polyphenolics due to its prevalence in plant physiology. While plant sugar residues can bind to any of the compound's aromatic carbons, this is not the most common form of attachment [4,5]. One important sub-class of polyphenolic compounds is flavonols. They are a well-known group of compounds with

pronounced antioxidant and ultraviolet light absorption properties [6]. Moreover, recently, their anticancer activity was also highlighted [7].

Up to now, many techniques have been employed to extract bioactive compounds from plants. However, there is still an increased interest in developing new, advanced procedures, to overcome the disadvantages of the existing techniques. One such new technique is the pulsed electric field (PEF). PEF is a green technique, relatively new that can be used to enhance the extraction processes [8]. It has been proven that by using PEF in the extraction process the total amount of specific compounds, especially polyphenols, can be increased [9,10]. The PEF typically is used before the main extraction step, as pre-treatment. Typically, short-duration pulses between (100 ns–1 ms) are used with a voltage between 1 kV and 3 kV [11]. So far, PEF has been used for the enhancement of the extraction of polyphenolics from citrus fruits [12], potato peels [13], *Sideritis* plants [14], rapeseed stems [13], etc. However, there were reported cases where the use of PEF did not increase the total polyphenol content (TPC) of other plants, such as *Thymus serpyllum* [15]. Therefore, its benefits should not be taken for granted, and further exploited on specific cases.

This work aims to examine the combinatorial effect of PEF (used as a pre-treatment step) along with ultrasound on the extraction of the phenolic compounds from *A. carnea*. The extraction was carried out using a simple, ultrasound-assisted procedure. Various solvent systems and PEF treatment times were examined prior ultrasound treatment, so as to examine whether the use of PEF is beneficial for polyphenol extraction.

2. Materials and Methods

2.1. Chemicals

Absolut ethanol, acetonitrile and formic acid were obtained from Carlo Erba (Val de Reuil, France). Glycerol anhydrous, gallic acid monohydrate and anhydrous sodium carbonate (>99%) were purchased from Penta (Prague, Czech Republic). Folin–Ciocalteu reagent was obtained from Panreac (Barcelona, Spain). Chemical standards for the HPLC-based determination of polyphenols (i.e., neochlorogenic acid, kaempferol 3-*O*-β-rutinoside, kaempferol 3-glucoside, quercetin 3-*O*-galactoside, quercetin and kaempferol) and polypropylene glycol were purchased from Sigma-Aldrich (Steinheim, Germany). A deionizing column was utilized to create the deionized water that was used in the experiments. All the chromatography solvents utilized were HPLC grade.

2.2. Plant Material Preparation and Extraction

Fresh leaves from a 20-year-old *A. carnea tree* were collected in Afidnes area (Attica, Greece, at according to Google Earth version 7.3.2.5776 Latitude: 38.181001 and Longitude: 23.849508) (the moisture of the leaves was calculated to be 75 ± 3%). Then, the leaves were washed thoroughly with deionized water and dried with paper towels. Next, 10 g of the leaves were cut into smaller pieces (<1 cm). Half of the leaves (5 g) were placed in the PEF treatment chamber, while the remaining quantity was left in the beaker (control sample). After 60 min, the PEF-treated leaves were removed from the chamber and placed in a glass beaker. Similarly, the non-PEF-treated leaves were also placed in a similar glass beaker. In both cases, 50 mL of an appropriate solvent was added [the solvents examined were: (I) deionized water, (II) 30% ethanol in water, (III) 30% glycerol in water and (IV) 30% polypropylene glycol in water]. The solid-to-liquid ratio employed herein resulted from our preliminary experiments, exhibiting a 18% increase compared to 1:5 ratio and 27% compared to 1:1 ratio. Next, the beakers were placed in an ultrasonic bath for 15 min. Then, the samples were centrifuged at 4000 rpm for 5 min and filtered using 0.45 μm filter papers.

The same procedure was followed to determine the effect of PEF treatment time, by altering the treatment time (30 min or 60 min). In this case, the solvent used for ultrasound-assisted extraction was 30% ethanol in water. Finally, the extracts were injected into the HPLC system and further analyzed.

2.3. Instrumentation

The PEF instrument used is given in detail in our previous studies [14]. Briefly, the system consisted of a high-voltage current generator (Leybold, LD Didactic GmbH, Hürth, Germany), a digital oscilloscope, a function/arbitrary waveform generator, and two stainless steel plates (10 cm long, 10 cm high) with 1 cm Teflon between them, for insulation. The pulse duration used to process the sample was 1 μs, the pulse frequency was 1 Hz, the electric field strength was 1 kV cm^{-1}, the waveform was a typical square wave, and the maximum delay was less than 20 ns. PEF parameters were selected based on our previous studies [10,13]. Ultrasound treatment of the samples was carried out in a Transonic 570/H (ELMA) unit, with a frequency of 35 kHz, and a high-frequency peak of 320 W.

The HPLC system used in this study was a Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany), connected to a diode array detector (Shimadzu SPD-M20A). A Phenomenex Luna C18 column (5 μm, 4.6 mm × 250 mm) (Phenomenex Inc., Torrance, CA, USA) was used as a stationary phase. The column was thermostated at 40 °C, during separation. The mobile phase consisted of (A) water containing 0.5% *v*/*v* formic acid and (B) a mixture of acetonitrile: water (60:40) containing 0.5% *v*/*v* formic acid. The gradient program was as follows: 5% B to 40% B in 40 min, then to 50% B in 10 min, and finally to 70% B in 10 min and kept constant for 10 more minutes. The flow rate was set at 1 mL min^{-1}. The total program run time was 70 min. The injection volume was 20 μL and injections were made using a rheodyne injector. Spectra were recorded between 220 and 360 nm. Identification of the compounds was carried out by comparing the retention time with that of standard compounds, as well as the absorbance spectra. For the quantification of the compounds, calibration curves were prepared with standard compounds and using the equations, the concentration of the compounds in the samples was determined.

2.4. Comparative Analysis of Total Polyphenol Content (TPC) of the Extracts

The TPC of the extracts was determined using the Folin–Ciocalteu assay, as previously described [16]. In brief, 0.1 mL of the diluted sample extract was mixed with an equal amount of the Folin–Ciocalteu reagent. After 2 min of incubation, 0.8 mL sodium carbonate aqueous solution (5% *w/v*) was added, and the solution was incubated for 20 min at 40 °C. Finally, the absorbance of the solution was measured at 740 nm. For the expression of the results in gallic acid equivalents (GAE), a calibration curve was prepared with gallic acid.

2.5. Statistical Analysis

All experiments were carried out three times and each sample was measured in triplicate, and therefore the results are expressed as mean values of all measurements, and variability of the results was expressed using the standard deviation (mean value ± standard deviation). Statistically significant differences between control samples and treated samples were evaluated with a *t*-test (after testing for normality of data with the Shapiro–Wilk test) for $p < 0.05$, using SPSS (version 26) (SPSS Inc., Chicago, IL, USA) software.

3. Results and Discussion

Since *A. carnea* can be commonly found in many cities as an ornamental plant, the valorization of its leaves in order to isolate bioactive compounds would be of high interest [1,2]. The most common method to prepare an extract is ultrasound treatment. However, PEF may have the potential to enhance the extraction yield, in an environmentally friendly way. To this end, the combinatory efficiency of PEF prior to ultrasound treatment was examined and optimized.

In order to assess the efficiency of the various solvents used for preparing the extracts of the plant, as well as whether the use of PEF as a pre-treatment step can further assist the extraction of bioactive compounds, the Folin–Ciocalteu assay was used. In this assay, the polyphenolic compounds react with the reagent and yield products that can be measured with photometers. In this context, the absorbance of the plant extracts prepared

with and without PEF, after reaction with the Folin–Ciocalteu reagent was used as a criterion to evaluate and compare the performance of the various conditions. Results are presented in Figure 1 and expressed as mg GAE per g of dry weight (mg GAE g^{-1} dw). As indicated by the results, the control samples (prepared without PEF pre-treatment) the optimum extraction solvent was the 30% ethanol in water mixture. The rest of the solvents, the glycerol-water and the propylene glycol-water mixtures yielded ~60% better results compared to plain water, but still less than the ethanol-water mixture. As regards the pre-treatment of the samples with PEF (60 min), it can be seen that, in the cases of water and ethanol-water mixture, the PEF pre-treated samples yielded higher results, that were found to be statistically significant for $p < 0.05$. The smaller increase (~4.6%) was recorded for the propylene glycol–water mixture, whereas when plain water was used a 18% increase was recorded. However, the most notable increase was recorded in the case of the ethanol–water mixture, where the yield of polyphenolic compounds was increased by 33%.

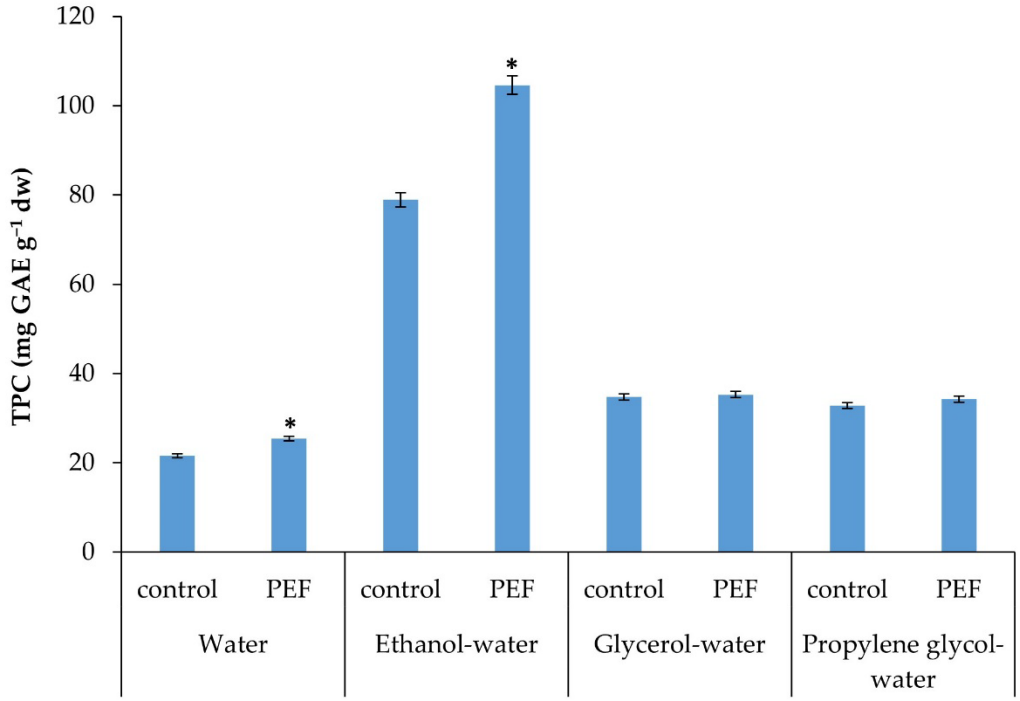

Figure 1. Total polyphenols, TPC (mg GAE g^{-1} dw), of the extracts after reaction with the Folin-Ciocalteu reagent; Statistically significant differences are denoted with an asterisk (*) for $p < 0.05$; Error bars denote the standard deviation of nine replicate analyses.

As regards the solvent used, they were selected due to their potential to extract phenolic compounds. Ethanol, not only is a commonly employed solvent for the extraction of polyphenolic compounds, but there are also many reports that highlight the superiority of hydroethanolic mixtures over water for the extraction of the compounds [17–19]. Glycerol is a naturally occurring compound that is also produced during biodiesel production, widely employed in food industry [20]. Due to its hydrophilic properties, it can be dissolved in water, and is considered as a more "green" alternative to ethanol. Glycerol has also been employed for the extraction of polyphenols from various plants, and in some cases, glycerol–water mixtures were found more efficient compared to ethanol-water mixtures [20–22]. Another polyol that has been studied for its potential for polyphenol extraction is propylene glycol. It is a safe compound for human consumption and has been used for the isolation of polyphenols from *Camellia* seeds [23], *Medicago lupulina* L. [24] and others [25]. Among the solvents used, the presence of ethanol was found to be beneficial. This is in accordance with previous reports [14,17–19,26,27]. A common explanation would be that the presence of ethanol in the extraction medium decreases the polarity of the solvent, rendering it more

suitable to extract less polar compounds. However, this is also the case with the examined polyols, rendering this explanation insufficient to explain the observed results. Moreover, it would also be expected that polyols would achieve a higher extraction efficiency, since they are capable of forming more hydrogen bonds with the polyphenols, compared to ethanol [22]. The polyol–water mixtures have increased viscosity, compared to plain water, since polyols, form an extensive network of hydrogen bonds, ascribing them high viscosity [20]. Thus, although the polyol–water mixtures are less polar than water and able to form more hydrogen bonds with the polyphenols, they have lower permeability to the plant tissue, due to their high viscosity. Ethanol addition in the water increases the permeability of cells, making easier the transfer of the compounds towards the extraction medium. Finally, it increases the heat conductivity of the solution, resulting in better heat transfer towards the cells and as a result, enhances the overall extraction procedure [28]. Regarding the use of PEF, there are previous studies that report increased extraction yield of polyphenols after treatment of the samples with PEF [14,29–31]. However, this is not always the case, since there are reports that the PEF treatment exhibits limited benefits for the overall extraction. For instance, in the study of Pollini et al. [32] the PEF pre-treatment of fresh apple pomace did not increase the extraction yield of polyphenol compounds. Similarly, in the study of Carpentieri et al. [33] the use of PEF prior to extraction of oregano and thyme with a hydroethanolic solution resulted in a nearly 7% increase in the TPC. Therefore, our results highlight the benefits of PEF usage, as a pre-treatment step to obtain extracts from *A. carnea* leaves that contain more polyphenols.

Based on the abovementioned results, we further examined whether a shorter time of PEF treatment (i.e., 30 min) would result in a similar content in polyphenols, compared to the treatments for 60 min, using the optimum solvent system. However, at this point, evaluation was based on individual polyphenolic components (presented in Table 1), and not on the TPC, in an effort to examine whether there are differences among the individual components, or if they all exhibit similar behavior. Representative chromatograms of the extract are presented in Figure 2.

Table 1. Content of specific polyphenols in the extracts (expressed as mean values ± standard deviation of nine replicate analyses) extracted with US only (without PEF) and after pretreatment with PEF for 30 or 60 min; Statistically significant differences are denoted with capital and small letters for $p < 0.05$.

Compound	Polyphenol Content (mg g^{-1})		
	Without PEF	**PEF 30 min**	**PEF 60 min**
Neochlorogenic acid	0.42 ± 0.03^{A}	0.52 ± 0.05^{a}	0.51 ± 0.06^{a}
Kaempferol 3-*O*-β-rutinoside	18 ± 1^{A}	22 ± 2^{a}	24 ± 3^{a}
Kaempferol 3-glucoside	28 ± 2^{A}	35 ± 3^{a}	40 ± 4^{a}
Quercetin 3-*O*-galactoside	0.23 ± 0.02^{A}	0.29 ± 0.03^{a}	0.36 ± 0.04^{a}
Quercetin	1.7 ± 0.2^{A}	2.4 ± 0.2^{a}	2.8 ± 0.3^{a}
Kaempferol	1.8 ± 0.2^{A}	2.6 ± 0.3^{a}	3.1 ± 0.3^{a}
Total identified	*50.2*	*62.8*	*70.7*

In the case of pretreatment with PEF for 30 min, the content of polyphenols in the extract was found to be 62.8 mg g^{-1}, whereas in the case of pretreatment with PEF for 60 min, the content of polyphenols in the extract was found to be 70.7 mg g^{-1}. No statistically significant differences ($p > 0.05$) were recorded for each examined compound, between the different samples, signifying that pretreatment with PEF for a shorter period is also able to achieve the same outcome, thus, further reducing the time and cost of the extraction process, without having a toll on the total extraction yield. Moreover, since no notable differences were recorded, it can be assumed that the PEF pretreatment assists the extraction process in a non-specific way, without being affected by the physicochemical properties of specific compounds (e.g., log K_{ow}).

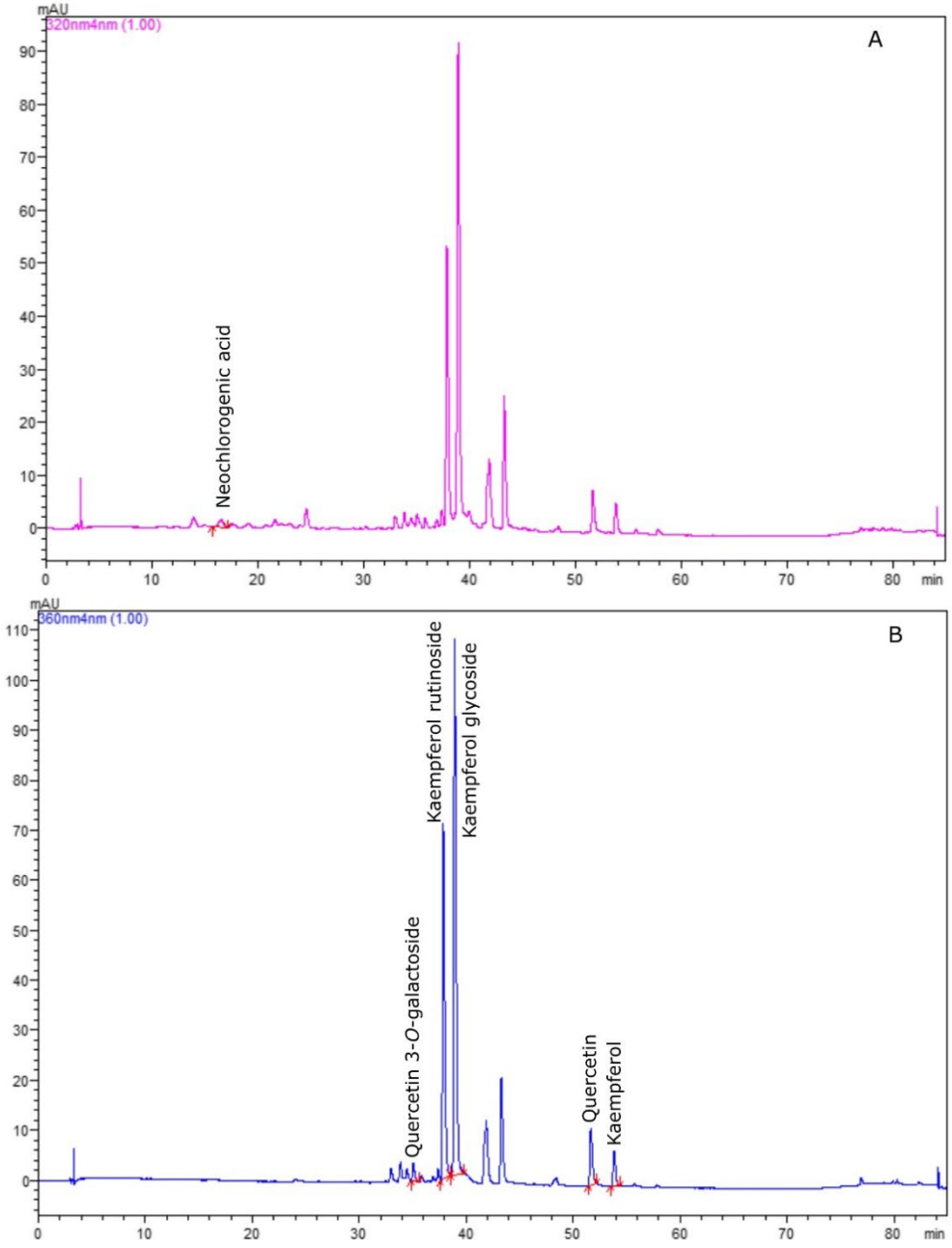

Figure 2. Representative chromatograms of the *A. carnea* extract at (**A**) 320 nm and (**B**) 360 nm.

4. Conclusions

In this study, the benefit of using PEF as a pretreatment, prior to US extraction of polyphenols from *A. carnea* was showcased. A significant enhancement of up to ~33% was recorded when PEF was employed. This percentage was achieved using an ethanol–water mixture as a solvent, which also enhanced the extraction of polyphenols, compared to plain water and polyol–water mixtures (i.e., glycerol and propylene glycol). The individual compounds examined exhibited a similar extractability for different PEF pretreatment times, suggesting a non-specific extraction mechanism, less dependent on the physicochemical properties of the specific polyphenols. The above data suggest that the proposed method can be used to enhance the content of polyphenols in the extracts, in a cost-efficient way.

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Conflicts of Interest: The authors declare no conflict of interest.

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