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Sustainable Extraction and Characterisation of Bioactive Compounds from Horse Chestnut Seed Coats for the Development of Bio-Based Additives

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Abstract: Background: To protect renewable packaging materials against autoxidation and decomposition when substituting harmful synthetic stabilizers with bioactive and bio-based compounds, extracts from *Aesculus hippocastanum L.* seeds were evaluated. The study objectives were to determine the antioxidant efficacy of bioactive compounds in horse chestnut seeds with regard to different seed fractions, improve their extraction, and to evaluate waste reuse. Methods: Different extraction techniques for field samples were evaluated and compared with extracts of industrial waste samples based on total phenolic content and total antioxidant capacity (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)). The molecular weight distribution and absorbance in ultraviolet range (UV) of seed coat extracts were determined, and the possibility of extracts containing proanthocyanidins was examined. Results: Seed coat extracts show a remarkable antioxidant activity and a high UV absorbance. Passive extractions are efficient and much less laborious. Applying waste product seed coats leads to a reduced antioxidant activity, total phenolic content, and UV absorbance compared to the field sample counterparts. In contrast to peeled seed extracts, all seed coat extracts contain proanthocyanidins. Discussion: Seed coats are a potential source of bioactive compounds, particularly regarding sustainable production and waste reuse. With minimum effort, highly bioactive extracts with high potential as additives can be prepared.

Keywords: European horse chestnut; seed coat; additive; antioxidant; proanthocyanidins; UV spectrum; extraction; size exclusion chromatography; polyphenols

1. Introduction

A growing population with an apparently even faster growing conscience about environmental issues and sustainability presents new challenges to the food and packaging industries in terms of eco-friendly, safe, and organic packaging systems that will not further contaminate oceans and the environment [1]. The demand for bio-products is increasing in agriculture [2], as well as for eco-power and energy-efficient devices [1]. Ecological conscience and the necessity of increased sustainability of packaging products made from common plastic or bio-plastic and improved by additives from renewable sources are ubiquitous. However, the instabilities of such materials occur due to photodegradation and microbial and oxidative stress, which can be mitigated by the application of proper additives. Without incorporation of such, the product undergoes undesired changes in

material properties, decreasing their stability and shelf life [3,4]. The global production volume of antioxidant additives in 2007 was 336.9 kt, with the majority of them being synthetic, petrol-based compounds, including the popular but potentially harmful additives butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [5–8]. Identifying and preparing bio-based bioactive additives to substitute petrol-based antioxidants is essential for producing packaging systems based on the concept of sustainable production and consumption [9]. This study is a precursor to the use of bio-based stabilizers in sustainable food packaging which, to the best of our knowledge, is a new approach.

On the current pharmaceutical market, use of extracts of European horse chestnut, or *Aesculus hippocastanum L.* (AEH) seeds (alias chestnuts), has already been established. Since the phytopharmaceutical industry focuses on ingredients found in the peeled seeds, the seed coats are usually discarded, providing an excellent, unexplored opportunity for by-product valorization. Pre-tests conducted as part of our previous research indicated a significant absorbance of AEH seed extracts in the ultraviolet range (UV). Expected substance groups in the extracts of seed coats are polyphenols, such as flavonols and condensed tannins alias proanthocyanidins (PAs), as both the mentioned structures as well as their glycosides were identified in Japanese horse chestnut seeds (*Aesculus turbinata BLUME* (AET)) and in AEH leaves [10–12]. Recently published preliminary tests support those findings, suggesting antioxidants, phenols, and a high UV-absorbing activity in the coats of AEH seeds [13].

In the context of their possible application as food contact materials, the harmlessness of the additives has to be evaluated. Fast and cost-effective extraction and implementation methods are equally important to create a competitive and attractive product. Therefore, ongoing optimization of extraction and analysis of the local horse chestnut species is essential. In this study, the secondary constituents of AEH seeds were extracted, and their total phenolic content (TPC), UV absorbance, and total antioxidant capacity (TAC) were measured and evaluated. As compounds with a higher molecular weight are more effective and less prone to migration [14], the molar mass distribution of extracts was determined. Extraction optimization is highly relevant for an efficient method; thus, extraction techniques based on using unprocessed, macroscopic samples were examined. Whereas such extraction methods are uncommon for most applications, they have been previously effective for extraction of PAs from different grape parts [15]. Thus, an efficient, simple, less elaborate extraction method was developed, optimized, and evaluated. Differences concerning the secondary ingredients and their amounts in different seed fractions (whole seed, peeled seed, seed coat) were investigated and evaluated. Additionally, seeds collected from the wild (field samples, FS) were compared to waste seed coats of the phytopharmaceutical industry (waste products, WP), and their applicability as additives was evaluated.

2. Materials and Methods

2.1. Chemicals and Instrumentation

For analysis, a Lambda 25 dual-trace spectral photometer (Perkin Elmer, Waltham, MA, USA) and, for size exclusion chromatography (SEC), a 1260 Infinity system with an 1100 Series column oven were used (Agilent, Santa Clara, CA, USA). The system is supplied with three SEC columns, including a pre-column (particle size: 5 µm) and two main columns (particle size: 5 µm; pore sizes: 1000 Å and 100,000 Å). All columns were produced by Polymer Standard Service (PSS; Mainz, Germany) and are equipped with a modified styrene-divinylbenzene copolymer network (SDV). The polystyrene standard kit used for SEC calibration was obtained from PSS (Mainz, Germany). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2(3)-tert-butyl-4-methoxyphenol (BHA), and dipotassium hydrogen phosphate were purchased from Alfa Aesar (Karlsruhe, Germany), whereas acetic acid, tetrahydrofuran (THF), Trolox, β-carotene, and 2,6-di-tert-butyl-4-methylphenol (BHT) were purchased from Bernd Kraft (Duisburg, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Cayman chemical Company (Ann Arbor, MI, USA), Sigma Aldrich (Darmstadt, Germany),

and ThermoFisher (Kandel) GmbH (Karlsruhe, Germany), respectively. Ammonium iron (III) sulfate dodecahydrate, butan-1-ol, concentrated hydrochloric acid, hexane, methanol, acetone, and agar were obtained from VWR International, Darmstadt, Germany. Dichloromethane, Folin-Ciocalteu phenol reagent, hydrogen peroxide, potassium dihydrogen phosphate, sodium hydroxide, sodium acetate, and nutrient broth for microbiology (based on 5 $g \cdot L^{-1}$ peptone from meat, 3 $g \cdot L^{-1}$ meat extract) were purchased from Merck KGaA, Darmstadt, Germany. Physiological saline solution and tryptone were purchased from Blank, Vörstetten, Germany and VWR International, Darmstadt, Germany.

2.2. Samples

As sample material, AEH seeds were collected from a single tree in Meckenheim, Germany (field samples (FS); coordinates: 50°36'7.3" N; 7°1'44.9" E) which was identified as AEH by scientific staff of the Faculty of Agriculture (University of Bonn, Germany). For pretests, further samples were collected and identified in further locations in Germany analogically. The quality of the samples collected from all locations meet the required standards; we focusef on FS from Meckenheim as the seeds were easy to collect and could be obtained in large quantities in one single harvest under similar conditions, promoting a more homogeneous sample material. In the following, the seed fractions are defined as whole seeds (ws) for whole seeds including the seed coat, peeled seeds (ps) for peeled seeds deprived of their coats, and seed coats (sc) for the dark brown seed coat alias seed shell only. The whole seeds were dried at 30 °C for 20 days until dryness (<10% water content). For 50 whole seeds, the average weight was determined, with 15 of those whole seeds being separated into peeled seeds and seed coats afterward to examine the weight ratio. Chopped AEH seed coats as phytopharmaceutical waste products (WP) were kindly provided by Finzelberg, Martin Bauer Group, Andernach, Germany.

2.3. Extraction

Different extraction techniques were applied for analyses. In a pretest, different extractants (water, methanol, water/acetone (1:1 *v/v*), methanol/acetone (1:1 *v/v*)) were used for the extraction of FS peeled seeds and FS seed coats to find a suitable extractant for the following research, evaluated by comparing the TAC. Water/acetone (1:1 *v/v*) was proven to be the most potent extractant regarding the extraction of substances from FS seed coats, whereas water was the most potent extractant for the FS peeled seed (Figure S1). Due to the peeled seed showing little antioxidant activity as described later, we focused on the extraction from seed coats. Thus, the extractant used in all further extractions was water/acetone (1:1 *v/v*) if not stated otherwise. Extractions took place at 22 °C.

For internally established extraction ("grinding extraction"), 200 mg ground sample was placed into a centrifuge tube. We pipetted 1 mL of the extractant onto the sample before mixing and centrifuging for 10 min. The supernatant was pipetted into a 5 mL volumetric flask and the previous steps were repeated twice more. All supernatants were combined and finally filled to 5 mL. The grinding extraction was applied to FS (ws, sc, and ps) and WP (sc) samples.

Finally, two variants of passive extraction setups were examined to evaluate a possible facilitation of the sample preparation process in practice.

For passive extraction of chopped seed coats, approx. 5 g of sample material with a size of approx. 5 mm were placed into 20 mL extractant and stored for a specific period in a closed container under exclusion of light. Also, a blank sample was prepared, stored for the maximum time period and subtracted from the results. This extraction was applied both to FS and WP chopped seed coats.

For passive extraction of FS whole seeds, three medium-sized seeds were cleaned with a brush and placed into a closed vessel. This corresponds to approx. 4.86 g seed coat on average. Then the vessel was filled with 67 mL extractant until the seeds were covered and stored for a specific period under exclusion of light. A blank sample was prepared accordingly.

2.4. Determination of Antimicrobial Properties

The antimicrobial activity of extracts of ground AEH seed coats was quantitatively analyzed by modifying the JIS Z 2801:2010 test for antimicrobial activity and efficacy [16]. *Staphylococcus aureus* (DSM No. 799) and *Escherichia coli* (DSM No. 1576) were applied as test organisms, and the extractant was used as a reference. The inoculum was prepared by transferring a frozen culture to 10 mL nutrient broth and incubating with the inoculum (37 °C, 24 h). According to the McFarland standard, the inoculum was adjusted in physiological saline solution with tryptone to a concentration of 108 colony forming units (CFU) mL^{-1} before being diluted in physiological saline solution with tryptone to a final concentration of 105 CFU·mL^{-1}. 1 mL inoculum was incubated (37 °C, 24 h) in a mixture of 9 mL nutrient broth and 1 mL extract (or solvent reference). Then, the samples were plated on plate-count agar by the drop plate technique. After incubation (37 °C, 24 h), viable counts were determined. A material is considered antimicrobial if the lg reduction calculated by Equation (1) is ≥2.0 after incubation [16]:

$$\text{lg reduction} = \text{lg}\,[c_{gew}(\text{reference}) \times (c_{gew}(\text{sample}))^{-1}] \quad (1)$$

Where c_{gew}(reference) is the arithmetic mean of bacterial counts of the reference 24 h after inoculation, and c_{gew}(sample) is the arithmetic mean of bacterial counts of the sample material 24 h after inoculation.

2.5. UV/Vis Spectrometry

The UV/Vis spectra of different extracts in appropriate dilutions were determined in the range of 260 to 800 nm. As diluting samples to different extents to obtain measurable and correct spectra was necessary, the results are displayed in relative absorbance units, considering the different dilutions to maintain comparability.

2.6. ABTS Radical Cation ($ABTS^{\bullet+}$) Scavenging Capacity Assay

The total antioxidant capacity (TAC) was determined at a wavelength of 660 nm according to the literature [17]. At least one blank sample per run was prepared. As the assay was calibrated using a Trolox solution, the TAC of the samples is given in mg of Trolox equivalents (Teq) per mg of extracted dried mass (DM) of the sample.

2.7. Folin-Ciocalteu Assay

To determine the TPC of the extracts, a modified Folin-Ciocalteu assay was conducted in centrifuge tubes [18,19]. First, 0.25 mL deionized water was mixed with the same amount of Folin-Ciocalteu reagent, and 0.25 mL of sample extract was added. At least one blank sample per measuring series was prepared. Then, 30 seconds after the sample was added and carefully mixed, 2.5 mL of 0.1% aqueous sodium hydroxide solution was pipetted into the centrifuge tube. The tube was capped, and the reagents were mixed. After exactly 30 min more, the absorbance of the sample was measured at the wavelength of 720 nm. For evaluation, the assay was calibrated with gallic acid. Therefore, TPC of the samples is given in mg of gallic acid equivalents (GAE) per g of extracted dried mass (DM) of the sample.

2.8. Size Exclusion Chromatography (SEC)

For SEC analysis, samples were prepared by evaporating seed coat extract under a nitrogen stream until complete dryness and subsequent solving in THF/water (20:1 *w/w*). This THF/water mixture is also the mobile phase for SEC measurement as it was used in the literature for polyphenols [20,21]. Further parameters were adjusted to a flow rate of 1 mL·min^{-1}, a sample injection volume of 100 µL, a measuring time of 30 min, and an isocratic elution at 35 °C. Detection was carried out by applying a UV detector measuring the absorbance at 280 nm. Molar mass calibration was conducted with a polystyrene standard kit.

2.9. Further Analyses

The modified Acid Butanol Assay was prepared according to the literature with analysis at a wavelength of 550 nm [22]. The assay is qualitative only as no calibration was prepared. For NMR analysis, the whole seed passive extract with an incubation time of 21 days was diluted with deuterated water and measured using an Avance III 600 NMR device (Bruker Corporation, Billerica, MA, USA).

3. Results and Discussion

3.1. Pre-Analyses

3.1.1. Seed Coat Ratio

Weighing of whole seeds resulted in an average weight of approx. 11.2 g per whole seed (standard deviation (SD): ± 1.6 g; n = 50). The average weight of the seed coat was 1.62 g (SD: ± 0.21 g; n = 15), and 9.65 g (SD: ± 0.83 g; n = 15) for the peeled seed for this average total weight, representing 14% seed coat per whole seed (SD: ± 1.2%; n = 15). The seed coat represents a relevant and potentially worthwhile source of resources. However, reference data concerning the mass ratio of AEH peeled seed and seed coat have not been published yet.

3.1.2. Determination of Antimicrobial Properties

Typically, the disc diffusion method is used for the determination of antimicrobial properties. However, to prevent potential issues due to macromolecular analytes that are less prone to diffusion, a modified Japanese Industrial Standard (JIS) method was applied as it is not dependent on the sample molecules successfully migrating into the agar. When determining the antimicrobial properties of AEH seed coat extracts obtained by grinding extraction, the arithmetic mean of bacterial counts of the reference for *S. aureus* is 8.0 lg CFU·mL^{-1} and for *E. coli* is 7.6 lg CFU·mL^{-1} after incubation. The average bacterial counts for *S. aureus* decreased to 1.8 lg CFU·mL^{-1} when applying FS extracts, a reduction of 6.2 lg units. For WP extracts, the average *S. aureus* bacterial counts diminished to 1.6 lg CFU·mL^{-1} (reduction: 6.4 lg units). For *E. coli*, no significant reduction was observed.

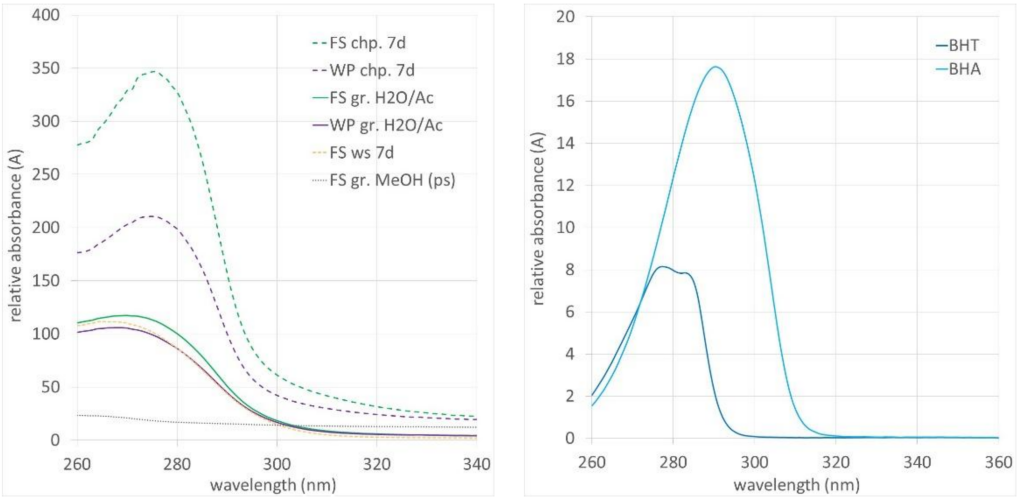
The results show that the gram-positive bacterium *S. aureus* is more sensitive against AEH seed coat extracts than the gram-negative bacterium *E. coli*. This observation of a stronger resistance of gram-negative bacteria against antimicrobial substances of plant origin is confirmed by the literature [23,24]. The effect is caused by differences in the cell wall construction of gram-positive and gram-negative bacteria [25,26]. However, AEH seed coats are a material worthwhile to study for sustainable additive production as a considerable antimicrobial effect of their extracts against *S. aureus* was proven.

3.1.3. UV Absorbance

Whereas the peeled seed extract only showed a low UV absorbance, the extracts of seed coats demonstrated a significant UV absorbance as shown in Figure 1a. All seed coat extracts showed a comparably insignificant absorbance in the visible range while significantly absorbing in the region below 310 nm with maxima at approx. 275 nm. As a high UV absorbance is desired for additives acting against photodegradation [27], these results are promising.

The highest absorbance was attained by the FS chopped seed coats with a maximum relative absorbance of approx. 346, followed by the WP chopped seed coats (max. absorbance 210) and the whole seed extract whose max. relative absorbance of approx. 110 was comparable to that of extracts based on grinding extraction. This indicates the applicability efficacy and competitiveness of these easy extraction methods. Furthermore, the WP seed coats absorbed less than their FS counterparts. However, unlike seed coats that were manually collected and separated from the seeds, WP seed coats include a significant amount of peeled seed fragments, which show a marginal absorbance only as shown in the previous before. Therefore, the lower UV absorbance of the waste seed coats is

reasonable. Additionally, the industrial pre-treatment of WP seed coats prior to analysis was unknown. For example, increased contact of the seed coats with extractants during washing steps might have reduced the amount of their ingredients. As known for other plant species, another factor influencing the seeds' properties is the location and climate surrounding of the trees [28]. The absorbance spectra are qualitatively comparable to those of commonly used stabilizing additives BHT and BHA, plotted in Figure 1b. Both BHT and BHA significantly absorb in the UV range below 300 and 320 nm with maxima at approx. 275 nm and 291 nm, respectively. Regarding the absorbance intensity, there is a factor of approx. 43 and 20 from BHT and BHA to FS chopped seed coats, respectively, based on a BHT or BHA solution with a concentration of 1.0 mg·mL^{-1}. Thus, 1 mL of this extract is theoretically capable of substituting approx. 43 mg BHT or 20 mg BHA with regard to UV absorbance. For WP chopped seed coats, the factors decreased to approx. 26 (BHT) and 12 (BHA), whereas the absorbance of the extract obtained by passive extraction of whole seeds resulted in factors of approx. 14 (BHT) and 6.3 (BHA). Therefore, the most potent extracts are based on chopped seed coats (FS, in particular). However, extract sustainability must be considered as using the slightly less potent extracts of the WP seed coats allows reuse of natural resources that otherwise would be lost. Additionally, the advantage of FS is likely to decrease when peeled industrially and less accurately. The application of chopped WP seed coats passively extracted, for example for seven days, is thus recommended.



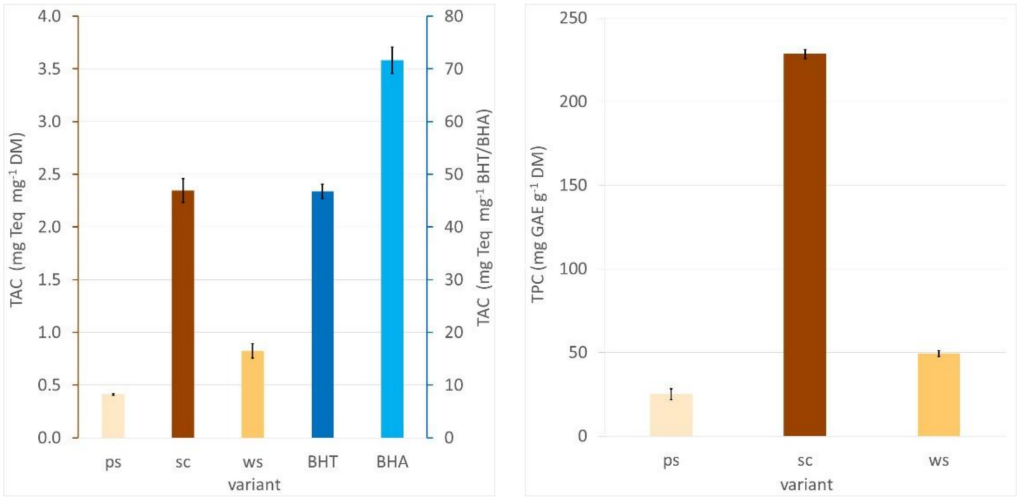
(**a**) (**b**)

Figure 1. (**a**) Average relative UV absorbance of *Aesculus Hippocastanum L.* seed coat and peeled seed extracts. Measurements were recorded in triplicate. No relevant absorbance above 360 nm was measured. FS: Field samples; WP: Phytopharmaceutical waste products; chp.: passive extraction of chopped seed coats; gr.: grinding extraction of seed coats (or peeled seeds (ps), if stated); ws: passive extraction of whole seeds; 7d: Extraction duration of 7 days; H_2O/Ac: Extractant water/acetone (1:1 *v/v*); MeOH: extractant methanol. (**b**) Average relative UV absorbance of BHT and BHA solutions. Measurements in triplicate. No relevant absorbance above 360 nm was measured. BHT: Butylated hydroxytoluene; BHA: Butylated hydroxyanisole. Solvent: methanol; concentration: 1.0 mg mL^{-1}.

3.1.4. Total Antioxidant Capacity (TAC) and Total Phenolic Content (TPC)

The comparison of the TAC and TPC of peeled seed and seed coat visualized in Figure 2 provides insight into the suitability of different plant parts for use as additives. With an average TAC of 1.98 mg Teq·mg^{-1} DM, the seed coat (sc) presented the highest value, followed by the whole seed (ws) with an average of 0.534 mg Teq·mg^{-1} DM and by the peeled seed (ps) with an average TAC of 0.319 mg Teq·mg^{-1} DM. Between the seed coat and whole seed, an approximate factor of 11 was observed, whereas the difference between whole and peeled seed was approximately a factor three. The average TPC of the seed coat extract was 234 mg GAE·g^{-1} DM, of the whole seed was

80 mg GAE·g^{-1} DM and of the peeled seed was 54 mg GAE·g^{-1} DM. The extracts of AEH seeds, in particular their coats, revealed high amounts of phenolic compounds and high antioxidant capacities, whereas the peeled seed extracts showed much lower amounts of phenolics and antioxidants. This also applies to FS seeds that were collected in other locations in Germany, separated in peeled seeds and seed coats and analyzed as a part of the pretests. AEH seed coat extracts in general thus meet the most important requirement for antioxidants. The findings correspond to the results of Vašková et al. who found phenolics to be one of the main substance groups found in AEH seeds [29]. However, further characterization of the ingredients as conducted during this study would be indispensable. Since the substances of interest are prevalent in the seed coats with the peeled seed containing relatively low amounts of antioxidants and phenolic substances, the peeled seed was widely neglected in this study. For the TAC and TPC, a recent short communication reported a mean TAC of 1.78 mg Teq·mg^{-1} DM and a mean TPC of 602 mg GAE·g^{-1} DM for AEH seed coat extracts [13]. In this study, a higher TAC and a lower TPC were determined. Since the extraction and TAC methods used by Makino et al. differ from the methods applied in this study, the comparability of the results is limited [13]. However, the results of Makino et al. support the findings presented in this study. Separation of seed and seed coat was conducted by Kimura et al., who also reported a high amount of PAs in AET seed coats with significantly higher amounts in the seed coat than in the peeled seed [10]. The measured TAC and TPC reasonably vary from the results of this study, presumably due to biological differences between European and Japanese horse chestnut and methodical deviations in extraction and analysis. The effects of different plant varieties and varying climate properties of different cultivation locations are known, too, most likely promoting differences in the results [28,30]. Compared to the TAC of synthetic antioxidants, which are provided in Figure 2a, factors of approx. 20 or 35 between the seed coat and BHT or BHA, respectively, were measured. Therefore, 20 mL or 35 mL of extracts obtained by grinding extraction could substitute 1 mg BHT or BHA, respectively, with regards to antioxidant efficacy.



(**a**) (**b**)

Figure 2. (**a**) Total antioxidative capacity (TAC) of synthetic antioxidants BHT and BHA and *Aesculus Hippocastanum L.* peeled seeds, seed coats, and whole seeds extracted by grinding extraction. Extracts: primary ordinate, given in shades of brown, measurements in triplicate; BHT/BHA: Secondary ordinate, depicts in shades of blue, six measurements. Standard deviation indicated by error bars. Teq: Trolox equivalents; DM: Dried sample mass; ps: Peeled seed; sc: Seed coat; ws: Whole seed; BHT: Butylated hydroxytoluene; BHA: Butylated hydroxyanisole. (**b**) Total phenolic content (TPC) of *Aesculus Hippocastanum L.* peeled seeds, seed coats and whole seeds extracted by grinding extraction. Measurements in triplicate, standard deviation indicated by error bars. GAE: gallic acid equivalents; dm: dried sample mass; ps: peeled seed; sc: seed coat; ws: whole seed.

3.1.5. Molar Mass Characterisation of AEH Seed Coat Extracts

The molar mass distribution of AEH seed coat extracts and the corresponding integral curve are plotted in Figure 3. The applied detection wavelength of 280 nm is considered characteristic for polyphenols [10]. Consequently, we assumed that the sample contained polyphenols in varying molecular sizes that are well displayed in the UV signal at 280 nm. The smallest 10% of the substances in the extract had a molecular weight below 1176 g·mol^{-1}, whereas the biggest 10% had a minimum molar mass of 4862 g·mol^{-1}. The number average molecular weight was 2097 g·mol^{-1}, and the molecular weight at the peak maximum was 2989 g·mol^{-1}. The weight average molecular weight of the compounds extracted from seed coats was determined to be 3095 g·mol^{-1}. This corresponds to approx. 10 condensed catechin molecules, neglecting possible condensations of other compounds. An average molecular weight of 1750 g·mol^{-1} was determined by Czochanska et al. for PAs extracted from ground whole AEH seeds by analyzing the terminal group ratio after thiolysis using ^{13}C NMR [31]. The shift to a higher number average molecular weight compared to those results is reasonable as they are based on extracting the whole seed, including the inner seed, which is known to contain high amounts of substances with a significantly lower molecular weight than the seed coats' PAs, possibly including smaller polyphenols [29,32]. With molar masses ranging from approximately 1100 to 2600 g·mol^{-1}, the masses obtained from AET seed coat extract analysis are lower than the results for the AEH counterparts [11]. However, the dimensions are similar. As a high molecular weight is preferred for substances used in food contact materials due to a reduced migration risk, the SEC results underline the potential of AEH seed coat extracts [33].

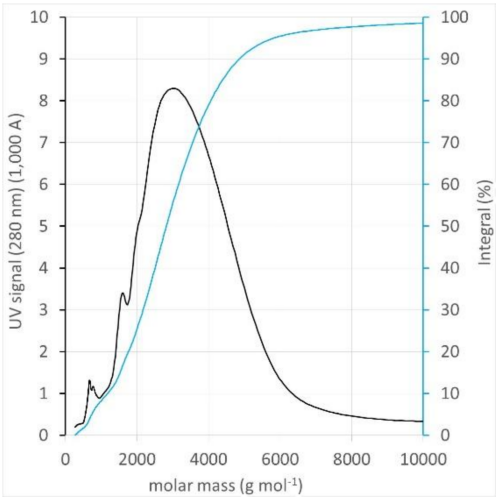


Figure 3. Evaluation of Size Exclusion Chromatography (SEC) analysis of *Aesculus Hippocastanum L.* seed coat extract. Primary ordinate: SEC chromatogram (signal of UV detector (UVD) at 280 nm), given in black and in thousands absorbance units; secondary ordinate: integral of SEC chromatogram, given in blue.

3.1.6. Further Analyses

Additional analyses, including 1H-NMR analysis and the Acid Butanol Assay, provided strong hints at different sugars and proanthocyanidins being present in the seed coats, inter alia supported by Kapusta et al. who found sugars in AEH seeds and Kimura et al. and Ogawa et al. proving proanthocyanidins being present in AET seed coats [10,11,34]. Again, this stresses the potential of AEH seed coats, as proanthocyanidins are classified as food-safe by the European Food Safety Authority [35].

Although the results suggest a separation of the seed fractions to prepare more potent extracts from the seed coat only, the drawbacks of the separation of seed and seed coat cannot be ignored. The manual separation is a time-consuming difficult task. When done automatically, separation will be less accurate, leading to loss of seed coat material and to incorporating parts of the significantly less

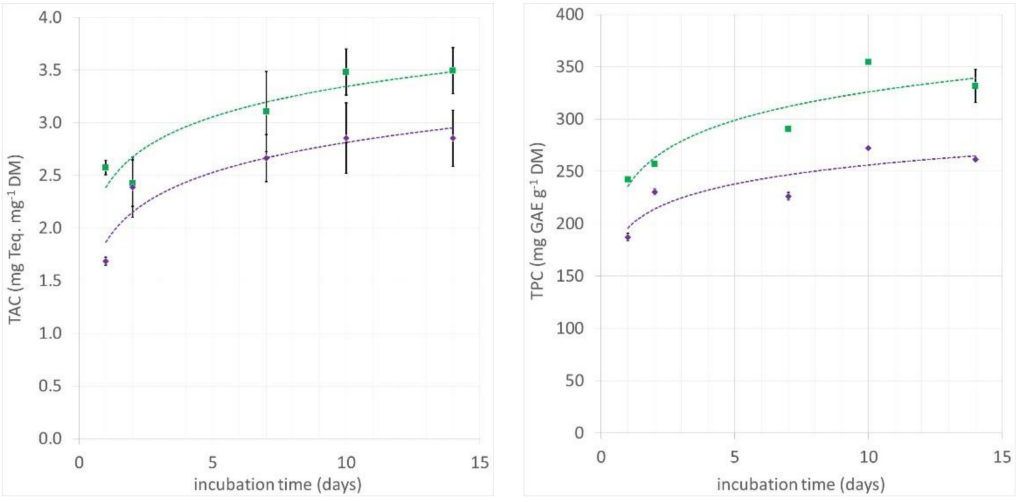
potent inner seed. Those issues might be mitigated by passive extraction setups, which are evaluated in the following.

3.2. Extraction Evaluation

3.2.1. Passive Extraction of Chopped Seed Coats

The passive extraction of seed coats is shown in Figure 4. Comparing the curves of FS and WP, a similar curve progression was noticed despite a deviation in the first data point. After two days of incubation, both sample types showed a TAC of approximately 2.4 mg Teq·mg^{-1} DM, following a steep increase. Afterward, the course was less steep, resulting in approximately 3.5 mg Teq·mg^{-1} DM for FS and 2.8 mg Teq·mg^{-1} DM for WP after an incubation time of 10 days with the values for 14 days barely diverging. Over the complete range, FS showed higher TAC values than WP. The corresponding TPCs had a similar progression with FS showing higher values over the complete course. Again, a rapid increase was noticed during the first days of incubation. In the following, a slow increase with a moderate scattering was noticed for both sample types, resulting in a maximum TPC after 10 days of 272 mg GAE·g^{-1} DM for WP and 355 mg GAE·g^{-1} DM for FS.

For the reasons discussed above, lower values for WPs are reasonable. However, the time-dependent courses are remarkably similar, so further noticeable deviations between FS and industrial WP seed coats did not occur during passive extraction. The slopes of both TAC and TPC matched the saturation curves. The TAC curves' rising slowed down after approx. seven days for both sample types. With a ratio of sample to solvent of approx. 1:4, this might be a sign of solvent saturation. The ratio is significantly smaller than that applied in the passive extraction of whole seeds (1:12) where no sign of stagnation was observed. This suggests that in the passive extraction setups, a solvent saturation takes place after 7–10 days at ratios between 1:4 and 1:12. Thus, in this setup, longer incubation times appear economically unreasonable. The comparison proves that the examined WP seed coats behave similarly to FS seed coats during extraction, except for the absolute starting concentration of phenolics and antioxidants, presumably for the same reasons as set out before.



(**a**) (**b**)

Figure 4. (**a**) TAC of passive extraction from *A. Hippocastanum L.* seed coats (chopped). Measurements in triplicate, standard deviation indicated by error bars. Teq: Trolox equivalents; DM: Dried seed coat mass. Field samples given in green (squares); waste products given in purple (diamonds). (**b**) TPC of passive extraction from *A. Hippocastanum L.* seed coats (chopped). Measurements in triplicate, standard deviation indicated by error bars. GAE: Gallic acid equivalents; DM: dried seed coat mass. Field samples given in green (squares); waste products given in purple (diamonds).

3.2.2. Passive Extraction of Whole Seeds

In the passive extraction setup using whole, unprocessed seeds, the TAC rapidly increased for the first seven days as shown in Figure 5a. After seven days, a TAC of 3.71 mg Teq·mg^{-1} DM was measured. Afterward, the average TAC value increased less steeply, but steadily, until it reached 7.05 mg Teq·mg^{-1} DM after 28 days of incubation. A similar TPC development of the extracts is illustrated in Figure 5b. After a rapid increase during the first seven days of incubation, a TPC of 343 mg GAE·g^{-1} DM was obtained. In the later course, a weak, but steady increase occurred up to a TPC of 596 mg GAE·g^{-1} DM after incubation for 28 days. The slopes of the extraction characteristics of whole seeds match a saturation curve that has not yet reached stagnation. As stated before, no sign of stagnation was observed during this experiment with a seed coat to solvent ratio of approx. 1:12, suggesting saturation at a ratio between 1:4 and 1:12.

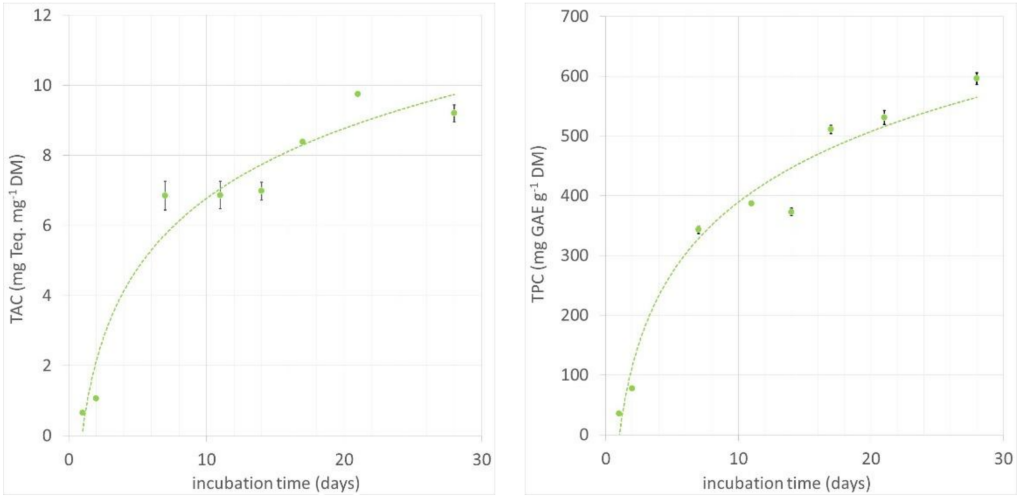


Figure 5. (**a**) TAC of passive extraction from whole *Aesculus Hippocastanum L.* seeds. Measurements in triplicate, standard deviation indicated by error bars. Teq: Trolox equivalents; DM: Dried whole seed mass. (**b**) TPC of passive extraction from whole *A. Hippocastanum L.* seeds. Measurements in triplicate, standard deviation indicated by error bars. GAE: Gallic acid equivalents; DM: dried whole seed mass.

Again, the rise in the TAC and TPC was steep at the beginning with a flattening after approx. 10 days, making 7–10 days the most efficient incubation time. Further increase in incubation time led to a relatively low extraction yield. For instance, quadrupling the incubation time from 7 to 28 days resulted in an average daily TPC increase of approx. 3.5% (approx. 1.6% for TAC). Those rates are marginal compared to the initial average daily increase rates from day 1 to 7 of 146% in TPC and 158% in TAC. In comparison with the extraction from ground seed coat material, the TAC after seven days' of incubation is higher than the TAC of the grinding extract by a factor of approx. 2.9, whereas the TPC was almost 1.5-fold higher for the passive extract. Referring to Makino et al. again, an extract with a two- to three-fold higher TAC was obtained in this study by passively extracting whole seeds for seven days [13]. However, the corresponding TPC of the extract was lower by a factor of approximately 1.8. As mentioned earlier, deviations between the extraction and analysis methods can cause variations in the results between the two research groups, enabling only limited comparisons.

3.2.3. Comparison of Passive Extractions

The TAC measurements of BHT and BHA allowed a comparison of the antioxidant efficacy of synthetic and bio-based stabilizers. Their values exceeded those of the whole seed passive extracts by factors of approx. 7 or 11, respectively. This means that approx. 7 mL or 11 mL extract, respectively, could substitute 1 mL of a BHT or BHA solution (concentration: 1 mg·mL^{-1}) or 1 mg of BHT or BHA.

Therefore, whole seed passive extracts are three times more effective than those prepared by grinding extraction with regard to TAC. For chopped seed coat passive extractions, the approximate factors for the FS and WP were higher and comparable to grinding extraction (FS: BHT: 15, BHA: 23; WP: BHT: 18, BHA: 27), presumably caused in part by solvent saturation. Although these factors suggest a relatively high amount of AEH seed extract would be needed for substitution, it is important to remember that no resource-consuming synthesis is necessary and preparation efforts can be minimized. Additionally, the chemical structure and molecular size of the extracted compounds suggest that they could be incorporated into the polymeric matrix successfully, allowing a significantly higher amount of extract to be used in the product. An advantage of the passive extraction of whole AEH seeds is that little sample preparation is needed and the produced extracts are more potent. The previously established grinding method requires a much higher amount of sample preparation and active work time. The long sample incubation period during passive extraction can be balanced by the high throughput possible. The application of both passive extraction setups thus has the potential to enable the exploitation of otherwise discarded, sustainable materials.

4. Conclusions

Especially by TAC and TPC, the separation of the seed and seed coat of the European horse chestnut AEH was proven to be an essential and powerful tool to increase yields of antioxidants in the extracts. However, to avoid an elaborate sample preparation, a simple yet potent extraction method was developed where solvent is poured over whole seeds. A variation of this method was tested with chopped seed coats in the form of phytopharmaceutical waste products, as they can easily be obtained and used in relatively large amounts, enabling high throughput extraction. For both setups, an incubation period of 7 to 10 days is considered most efficient, yielding in very high amounts of TPC and TAC.

Phytopharmaceutical waste products have been proven to be well-suited as a source of additives. Application of these chopped seed coats is a convenient method of waste reuse, having advantages both from ecological and economical points of view. This also applies to using unused seeds from AEH trees, e.g., in urban environments. This new by-product valorization approach suits the sustainable concept of an environmentally friendly product from regional sources in both cases. Besides extraction optimization and conception, molar mass characterization of the extracted components was conducted in investigating the field samples. All tested seed coat extracts contained macromolecular substances that are likely to be proanthocyanidins, and the peeled seed was found to contain no significant amounts. The weight average molecular weight of the substances in the seed coat extracts was determined to be approx. 3095 $g \cdot mol^{-1}$. The high molecular weight of PAs diminishes the risk of migration when applied in packaging, potentially making AEH seed coat extracts an excellent additive for food contact materials. The applications of such seed coat extracts will be further examined; compounds of lower molecular weight will be characterized as part of upcoming migration studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-9276/8/2/114/s1, Figure S1: Solvent-dependent total antioxidative capacity (TAC) of peeled seeds (ps), seed coats (sc) and whole seeds (ws) from *Aesculus hippocastanum L.* extracted by grinding extraction. Measurements in triplicate, standard deviation indicated by error bars. Teq.: Trolox equivalents; DM: dried sample mass.

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