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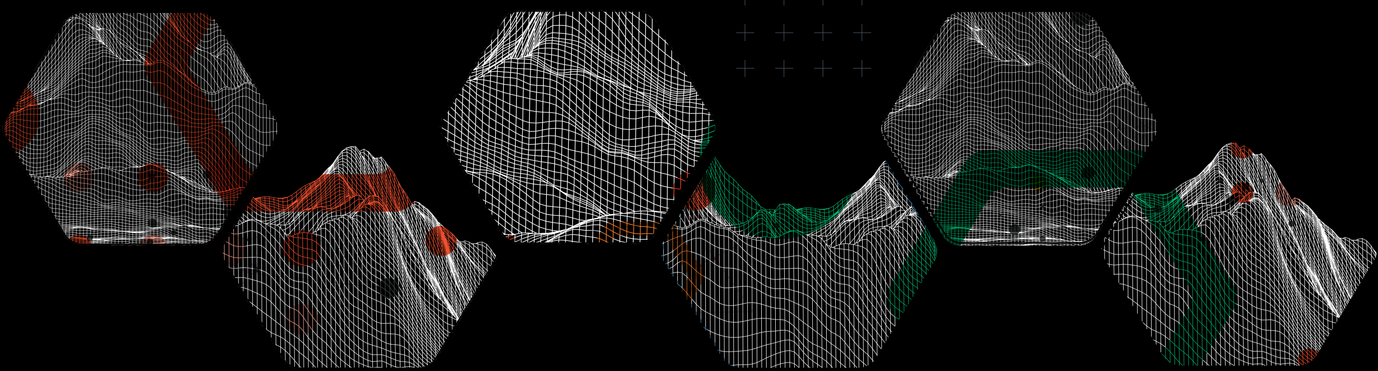

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APRESENTAÇÃO

Esta obra constituiu-se a partir de um processo colaborativo entre professores, estudantes e pesquisadores que se destacaram e qualificaram as discussões neste espaço formativo. Resulta, também, de movimentos interinstitucionais e de ações de incentivo à pesquisa que congregam pesquisadores das mais diversas áreas do conhecimento e de diferentes Instituições de Educação Superior públicas e privadas de abrangência nacional e internacional. Tem como objetivo integrar ações interinstitucionais nacionais e internacionais com redes de pesquisa que tenham a finalidade de fomentar a formação continuada dos profissionais da educação, por meio da produção e socialização de conhecimentos das diversas áreas do Saberes.

Agradecemos aos autores pelo empenho, disponibilidade e dedicação para o desenvolvimento e conclusão dessa obra. Esperamos também que esta obra sirva de instrumento didático-pedagógico para estudantes, professores dos diversos níveis de ensino em seus trabalhos e demais interessados pela temática.

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03

Effect of extraction conditions on flavonoids, tannins, antioxidant and antimicrobial activities of chestnut outer shells (*Castanea sativa* Miller)

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ABSTRACT

Background. During the chestnut processing, are generated high amounts of by-products such as chestnut outer shells. They are a promising source of bioactive compounds. This study aimed to evaluate the presence of bioactive compounds in chestnut outer shells (*Castanea sativa* Mill.), to valorize this by-product. **Material and methods.** Several extraction procedures were used, specifically different solvents (water, ethanol:water (80:20) and acetone:water (80:20)), combined with two extraction methods (stirring (ST) and ultrasounds (US)) and extraction times. Flavonoids, hydrolizable and condensed tannins, antioxidant activity, anti-microbial activity were determined in the extracts. HPLC-PDA analysis was made to identify some phenolic compounds. **Results.** The acetone:water (80:20) mixture was the best solvent to extract flavonoids and condensed tannins. With this solvent, the highest total reducing capacity, DPPH radical scavenging activity, and reducing power were also obtained. On the contrary, the most suitable extraction solution for hydrolysable tannins was ethanol:water (80:20). Gallic, (+)-catechin, and tannic acids were detected by HPLC-PDA. Only the extracts obtained by the US exhibited antimicrobial activity. **Conclusion.** It was stated that chestnut outer shells have high amounts of bioactive compounds. In the extractions, the solvent plays a higher role than the method applied.

Keywords: Outer Shell, Chestnut, Bioactive Compound, Extraction Method, Solvent.

INTRODUCTION

Portugal is one of the most important chestnut (*Castanea sativa* Mill.) producers in Europe, representing almost 12% of European production (FAOSTAT, 2022).

These nuts can be consumed as fresh, frozen, and other processed forms, being roasting the thermal treatment most used in-house and industrially. High amounts of by-products are generated during the peeling process of the chestnuts, representing around 20% of the total fruit weight. Their primary use has been as fuel (HU; YANG; CHANG, 2021).

The increasing demand for biologically active molecules has stimulated the search for natural sources of these compounds. It is recognized that polyphenols can reduce the risk of several diseases, including certain types of cancer, cardiovascular and neurodegenerative diseases, diabetes, and inflammation due to their antioxidant and antimicrobial activities (PINTO *et al.*, 2021; PANZELLA *et al.*, 2020; HU; YANG; CHANG, 2021). Furthermore, these valuable compounds are very important in the food industry because they can increase food stability by preventing lipid peroxidation (PINTO *et al.*, 2021).

Polyphenols may remain in the by-products after industrial processing due to their considerable molecular weight, covalently linkage, and complex bonds with other plant metabolites (AIRES; CARVALHO; SAAVEDRA, 2016). The main phenolic compounds extracted from chestnuts' shells are phenolic acids, flavonoids and tannins (VÁZQUEZ *et al.*, 2009; DE VASCONCELOS *et al.*, 2010; VIUDA-MARTOS *et al.*, 2011; HAM; KIM; LIM, 2015; SQUILLACI *et al.*, 2018; VELLA *et al.*, 2018, VELLA *et al.*, 2019; and CACCIOLA *et al.*, 2019). In particular, tannins are polyphenolic secondary metabolites of plants, that can be divided into hydrolyzable and condensed tannins (proanthocyanidins) (BRAGA; RODRIGUES; P.P. OLIVEIRA, 2015). Generally, the measurement of the first ones is more habitual than that of the last ones because the quantification method of condensed tannins is more laborious and time-consuming.

The solubility of polyphenols is influenced by the polarity of the solvents, extraction time and temperature. Different solvents have been used in the extraction processes employed to chestnut by-products (FERNÁNDEZ-AGULLÓ *et al.*, 2014; VÁZQUEZ *et al.*, 2008). Aqueous organic solvents have been reported to be better solvents than pure ones (VÁZQUEZ *et al.*, 2008), as the combination may facilitate the extraction of chemicals that are soluble in water and/or organic solvents (OROIAN; ESCRICHE, 2015). The most common extraction method applied has been stirring; however, other faster extraction techniques, such as ultrasounds (US) or microwave, may be used. In chestnut's shell extractions, of our knowledge, the US was used only by CACCIOLA *et al.* (2019), SILVA *et al.* (2020) and ŽIVKOVIĆ *et al.* (2010), who used one kind of solvent and extraction time, namely, water (60ºC, 60 min), ethanol (stirring for 2 h + US for 5 min) and ethanol 50% (v/v) (30 min), respectively.

Thus, the present work aimed to study the role of the extraction conditions. For these purpose, three different solvents (water, ethanol:water (80:20 v/v), and acetone:water (80:20 v/v)), two extraction methods (US and stirring), and extraction times were evaluated regarding the polyphenolic compounds isolated from chestnut outer shells after hot air drying, including condensed tannins. Different compounds were quantified, namely, flavonoids, hydrolyzable and condensed tannins, as well as the antioxidant activity, determined by the total reducing capacity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power assays. Additionally, some individual compounds were tentatively identified by HPLC-PDA detection. The antimicrobial activity of the obtained extracts was also screened using different target microorganisms. Thus, with this work, it is intended to valorize this significant by-product (chestnut outer shell) in line with the circular economy principles.

■ MATERIALS AND METHODS

Chestnut samples

The chestnut fruits (*Castanea sativa* Mill.) of the cultivar Longal were collected in October 2020 in orchards located in Espinhosela - Bragança, northeast of Portugal. The chestnuts were hot air dried in an oven (Series 9000, Scientific) at 180°C for 30 min, to mimic the common roasting method applied at home. Subsequently, the fruits were peeled, and the pellicle was separated from the outer shell. The outer shell was milled (A11 basic, IKA), weighed, frozen and freeze-dried (Coolsafe, Scanvac) for 72 h. After this period, it was weighted again and stored at -20°C in a dry and dark environment in closed flasks until extraction.

Chestnut extract preparation

The powdered samples of the chestnut shell were extracted with three different solvents: ultrapure water, ethanol:water 80:20 (v/v), and acetone:water 80:20 (v/v) (decreasing polarity). Among solvents, ethanol possesses the “GRAS” status (Generally-Recognized-As-Safe, according to American Food and Drug Administration), which allows it to be used in food industry. Ten grams of sample were mixed with 300 mL of solvent. Each solvent solution was subjected to two extraction methods – ultrasounds in a ultrasonic bath with a frequency of 45 kHz (Ultrasons-H, Selecta) and orbital stirring (SI4-2, Shel Lab) - at different extraction times, namely ultrasounds (US) for 1 and 5 min and orbital stirring (ST) for 30 and 60 min. Both types of extraction were conducted at room temperature (20°C). The extraction times were chosen taking into account the conditions applied by CACCIOLA *et al.* (2019), SILVA *et al.* (2020) and ŽIVKOVIĆ *et al.* (2010), as mentioned previously, but only one solvent was studied by these authors.

After extractions, the solutions were filtered, and the solvents were removed by vacuum distillation (R-114, Buchi) at 40°C. Then the samples were frozen and freeze-dried to determine the extraction yields. Afterwards, all extracts were redissolved in the corresponding solvent at 10 mg/mL and stored frozen at – 20°C.

Flavonoids

The total flavonoid contents were determined by a colourimetric assay based on the formation of a flavonoid–aluminium compound, according to FERNANDES *et al.* (2015) and VIUDA-MARTOS *et al.* (2011).

Hydrolyzable tannins

The hydrolyzable tannins contents were determined by applying the procedure described by FERNANDES *et al.* (2015).

Total condensed tannins

Total condensed tannins were determined using the acid butanol assay described by DE VASCONCELOS *et al.* (2010), with some modifications. Briefly, 75 µL of the extract was added to 1.4 mL of HCl:1-butanol (5:95 v/v) in a screw cap tube, sealed and heated at 95°C for 2 h. The samples were centrifuged at 12,300´*g* for 10 min. The absorbance of the supernatants was measured at 555 nm (V-530, Jasco). A partially-purified condensed tannin extract was previously prepared to construct the calibration curve. The preparation of the standard is described in the next section.

Preparation of the condensed tannin standard from chestnut shell Six sub-samples were prepared by weighing (6 × 50 mg) of chestnut shell into a 10 mL screw cap glass tube. To each tube, 5 mL of 70% methanol was added. The tubes were sealed, placed in a water bath at 70°C for 1 h (stirring each 10 min) and centrifuged at 2,057 ´*g*, for 10 min. The supernatants were recovered from the six tubes and mixed. This mixture was evaporated under vacuum until a volume of approximately 3 mL.

A silica solid phase-extraction column (Chromabond C18 EC-Endcapped, 6 mL, 500 mg adsorbent) was pre-washed with 20 mL of 100% methanol, followed by 40 mL ultra-pure water (Direct-Q 3UV, Millipore). The sample was loaded onto the column. The first elution, 15 mL of 20% methanol/80% dichloromethane, was discharged. The condensed tannins were eluted with 20 mL of 100% methanol. This fraction was collected in a pre-weighed flask and evaporated under vacuum. Afterwards, the dry sample was re-suspended in 100% methanol to give a 5 mg/mL stock solution. This solution was used to construct the condensed tannin calibration curve.

Reversed-phase HPLC analysis

Some individual phenolic compounds present in the extracts were tentatively identified by HPLC-PDA detection, using a Nucleosil® C18 analytical column (15 cm × 4.6 mm, 5 µm particle size), working at room temperature. The injected sample volume was 20 µL. A Jasco MD-4010 photodiode array detector was used, being the absorbance monitored at different wavelengths. The solvent flow rate was 1 mL/min, and the mobile phase consisted of Solvent A (water:acetonitrile, 90/10, at pH= 3 adjusted with acetic acid) and Solvent B (water:acetonitrile, 10/90, at pH= 3). A linear gradient method was used, starting with 100% solvent A and ending with 100% solvent B. Each run lasted 40 min. The pure standards used were gallic acid, catechin, ellagic acid, ferulic acid, tannic acid and rutin.

In vitro Antioxidant Activity

Total reducing capacity The total reducing capacity of the extracts was determined by the Folin-Ciocalteu method, based on the procedure described by SINGLETON & ROSSI (1965) with some modifications. A volume of 0.5 mL of sample was mixed with 0.5 mL of Folin-Ciocalteu's phenol reagent. After 3 min in the dark, 0.5 mL of saturated sodium carbonate solution and 3.5 mL of distilled water were added to the mixture. The reaction was kept in the dark for 90 min, after which the samples were centrifuged for 5 min at 3,214 ´*g*. The absorbance was read at 725 nm (V-530, Jasco). A blank prepared by substituting the extract solution with the solvent was used for background subtraction. Gallic acid was used for constructing the standard curve (10 - 300 mg/L), being the results expressed in milligrams of gallic acid equivalents (GAEs) per gram of extract.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity DPPH radical-scavenging activity was determined following the procedure described by FERNANDES *et al.* (2015).

Reducing power The reducing power of the extracts was determined as described by FERNANDES *et al.* (2015).

Antimicrobial activity

The disc diffusion method was used as a screening test for antimicrobial activity, following the procedure described by ŽIVKOVIĆ *et al.* (2010). The microorganisms were obtained from the American Type Culture Collection (ATCC), and cultured aerobically at 37°C (FL115, Binder) in a nutritive agar medium (NA) for bacteria, and at 30°C (SI4-2, Shel Lab) in Sabouraud dextrose agar medium (SDA) for yeast.

In a test tube with 3 mL NaCl 0.9% (w/v), 2 or 3 colonies of microorganisms were placed and incubated for 2-3 h, at 37°C. After this, the turbidity was adjusted spectrophotometrically (V-530, Jasco) to 0.8-1.0 at 625 nm with NaCl 0.9% (w/v) solution.

Sterilized filter paper disks (6 mm diameter) were impregnated with extracts sample (15 µL of 10 mg/mL of *C. sativa* shell extracts). The disks were placed on Mueller Hinton agar plates, which had been previously inoculated with Gram-positive (*Staphylococcus aureus, Bacillus subtilis, B. cereus, Enterococcus faecalis*), Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Proteus mirabilis, Klebsiella pneumoniae*) bacteria, and yeast (*Candida albicans*). After incubation for 24 h at 37°C, the inhibition zone diameters (IZ including disk) were measured and expressed in mm. Each test was made in triplicate.

Statistical analysis

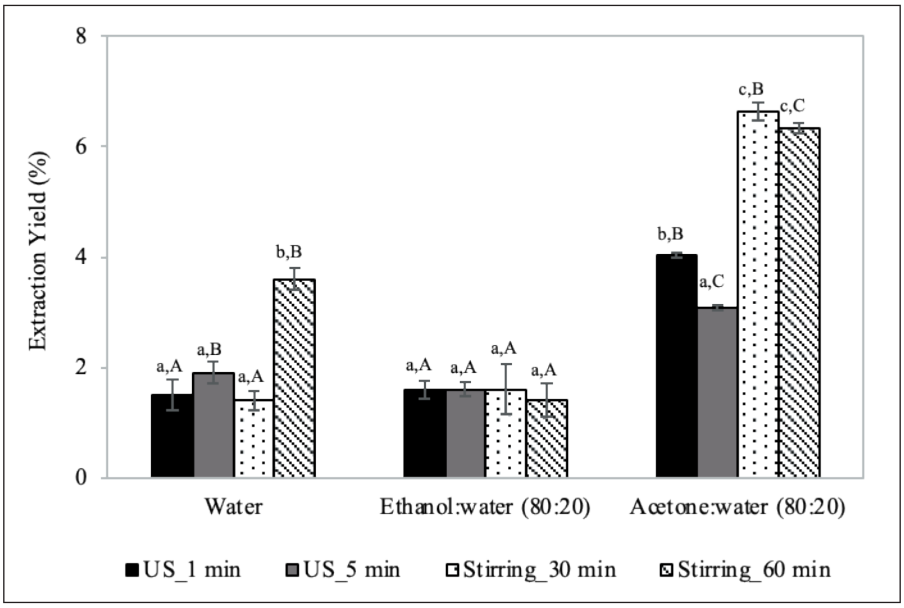
All determinations were made in triplicate. Data were reported as mean ± standard deviation. Statistical analysis of the results was performed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk test verified the normality of the data, and Levene's test verified the homogeneity of the variances. As the data followed a normal distribution, analyses of variance (ANOVA) or ANOVA Welch were used to detect the differences between different samples for all assays. ANOVA was applied when the homogeneity of variances was observed, while ANOVA Welch was applied for the other cases. Additionally, post hoc multiple comparisons of the means were performed according to the Tukey or Games-Howell tests, when it was observed homogeneity and non-homogeneity of variances, respectively. In all cases, $p < 0.05$ was accepted as denoting significant differences. A Principal Component Analysis (PCA) was also performed to evaluate the effect of the applied conditions on the composition of the extracts and differentiate them. The number of components to keep for data analysis was assessed by: (i) the respective eigenvalues (must be >1); (ii) Cronbach's α parameter (that should be positive); and (iii) the total percentage of variance (that should be as high as possible) explained by the number of components selected.

RESULTS AND DISCUSSION

Extraction yield

Concerning the extraction yields, these were usually low (< 7%) (Figure 1).

Figure 1. Extraction yields of extracts obtained from chestnut shells after applying different solvents and extraction methods (US – Ultrasounds and Stirring).



Different lowercase letters mean significant differences between treatments for a given solvent (p<0.05) and different uppercase letters mean significant differences between solvents for a given treatment.

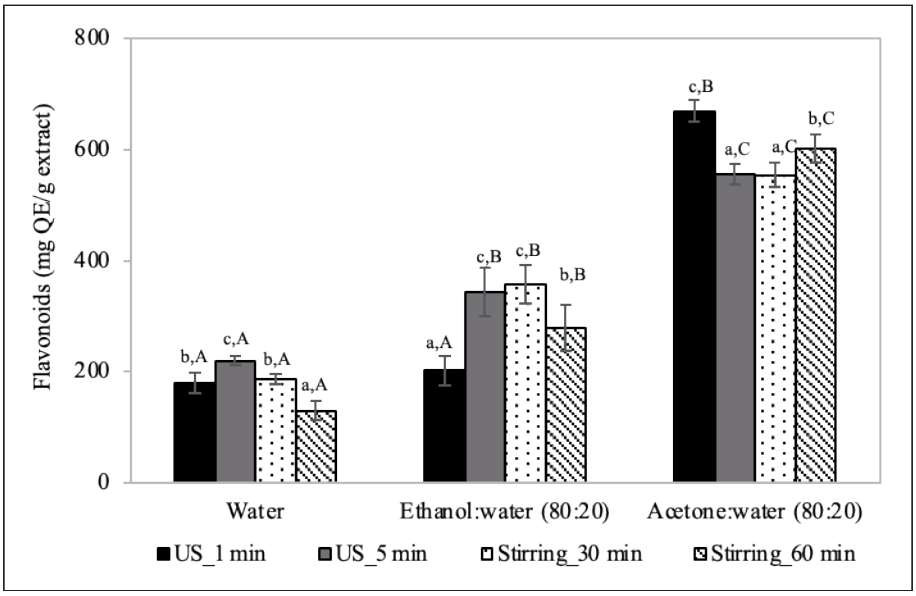
Nevertheless, the highest values were obtained with acetone:water (80:20), the solvent with the lowest polarity. Moreover, the methods that originated the highest extraction yields were stirring for 30 and 60 min. The ultrasounds for 1 and 5 min extracted only approximately half of the stirring yields. On the contrary, ultrasounds for 1 and 5 min caused extraction yields with ethanol:water (80:20) and water similar to those obtained with stirring for 30 min. So, a reduction in the extraction time of at least six times was achieved with ultrasounds compared with stirring when applying these green solvents. Nevertheless, stirring with water for 60 min almost doubled the extraction yield compared with the other methods.

Our results agree with those of MELISSA NAZZARO *et al.* (2012), who extracted chestnut shells with methanol, methanol:water (70:30, v/v) and water at room temperature, during five days, under constant stirring. The lowest extraction yield was also obtained when water was used as a solvent. Also, FERNÁNDEZ-AGULLÓ *et al.* (2014) obtained the lowest extraction yield with water at 25°C, but when they increased the temperature of the extractions to 75°C, higher extraction yields were obtained. The use of aqueous solutions has been recommended as VÁZQUEZ *et al.* (2008), who concluded that the highest extraction yield was obtained with water compared with other organic solvents such as methanol, ethanol, ethyl acetate, acetone, and *n*-hexane. These researchers concluded that the extract yield increased with the polarity of the solvent. Still, the total extraction yield must always be analyzed together with other bioactive compounds yields to optimize the process.

Flavonoids

The total flavonoids contents in dry extracts obtained from chestnut shells after applying different solvents and extraction methods (US and ST) are shown in Figure 2.

Figure 2. Total flavonoids determined in dry extracts obtained from chestnut shells after applying different solvents and extraction methods (US – Ultrasounds and Stirring).



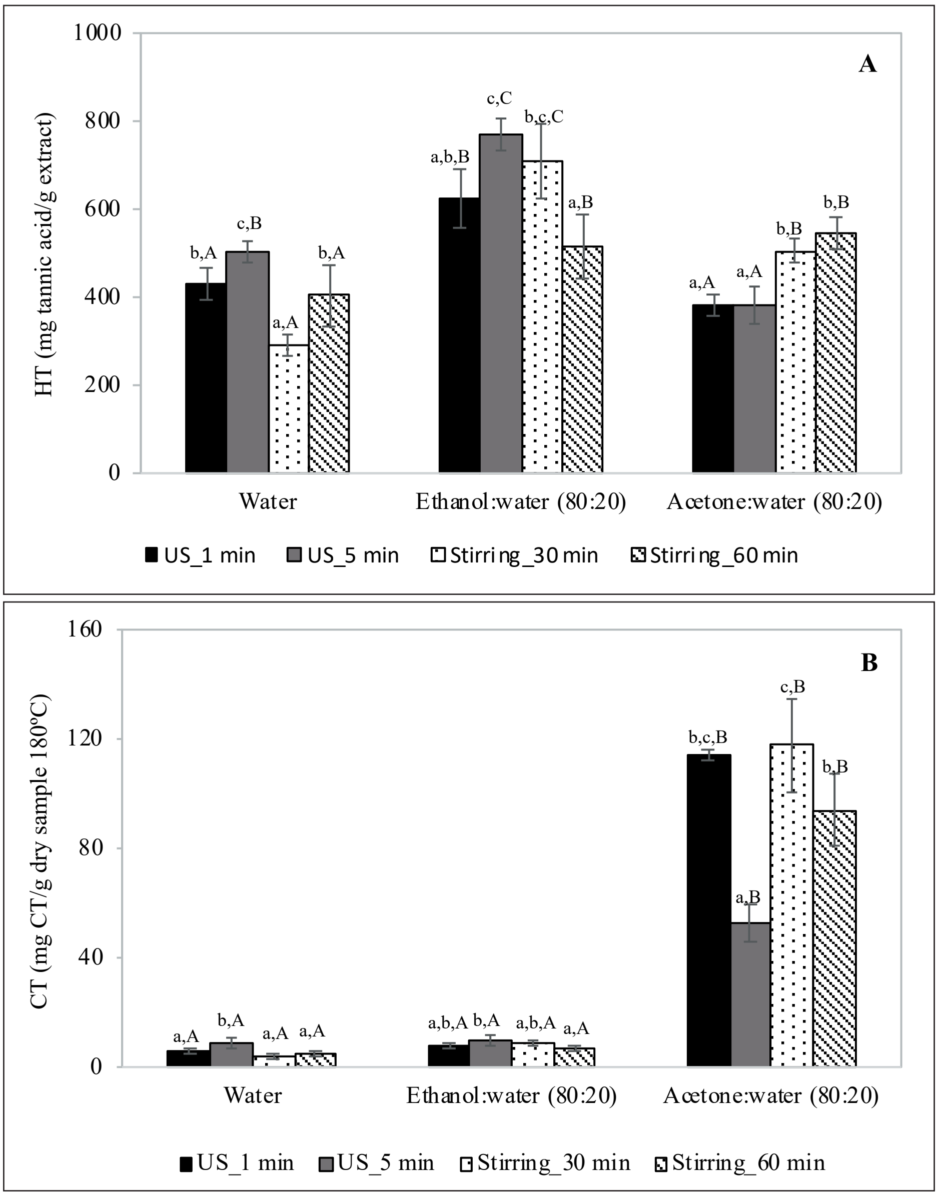
Different lowercase letters mean significant differences between treatments for a given solvent ($p<0.05$) and different uppercase letters mean significant differences between solvents for a given treatment.

For ethanol:water (80:20), the best results were found in US for 5 min and ST for 30 min. For water, the best result was obtained again with US for 5 min. However, ST for 30 min led to a smaller flavonoids extraction but equivalent to the treatment of US for 1 min. On the contrary, ST for 60 min originated the lowest total flavonoids content.

Hydrolisable tannins

Concerning tannins (Figure 3), the hydrolyzable tannins (HT) were detected in high amounts in the outer shell of chestnuts (291±24 to 771±38 mg tannic acid/g extract).

Figure 3. Hydrolisable (HT) (A) and Condensed (CT) (B) tannins determined in dry extracts obtained from chestnut shells after applying different solvents and extraction methods (US and Stirring).



Different lowercase letters mean significant differences between treatments for a given solvent (*P*<0.05) and different uppercase letters mean significant differences between solvents for a given treatment.

Ethanol:water (80:20) was the best solvent to extract these compounds. The highest HT concentrations were observed with US for 5 min with this solvent, closely followed by ST for 30 min. With water, the maximum HT level was determined by the US for 5 min. On the contrary, with acetone:water, the highest concentrations of HT were obtained with ST for 30 and 60 min, suggesting that, with this solvent, higher extraction times must be applied to extract these compounds.

Condensed tannins

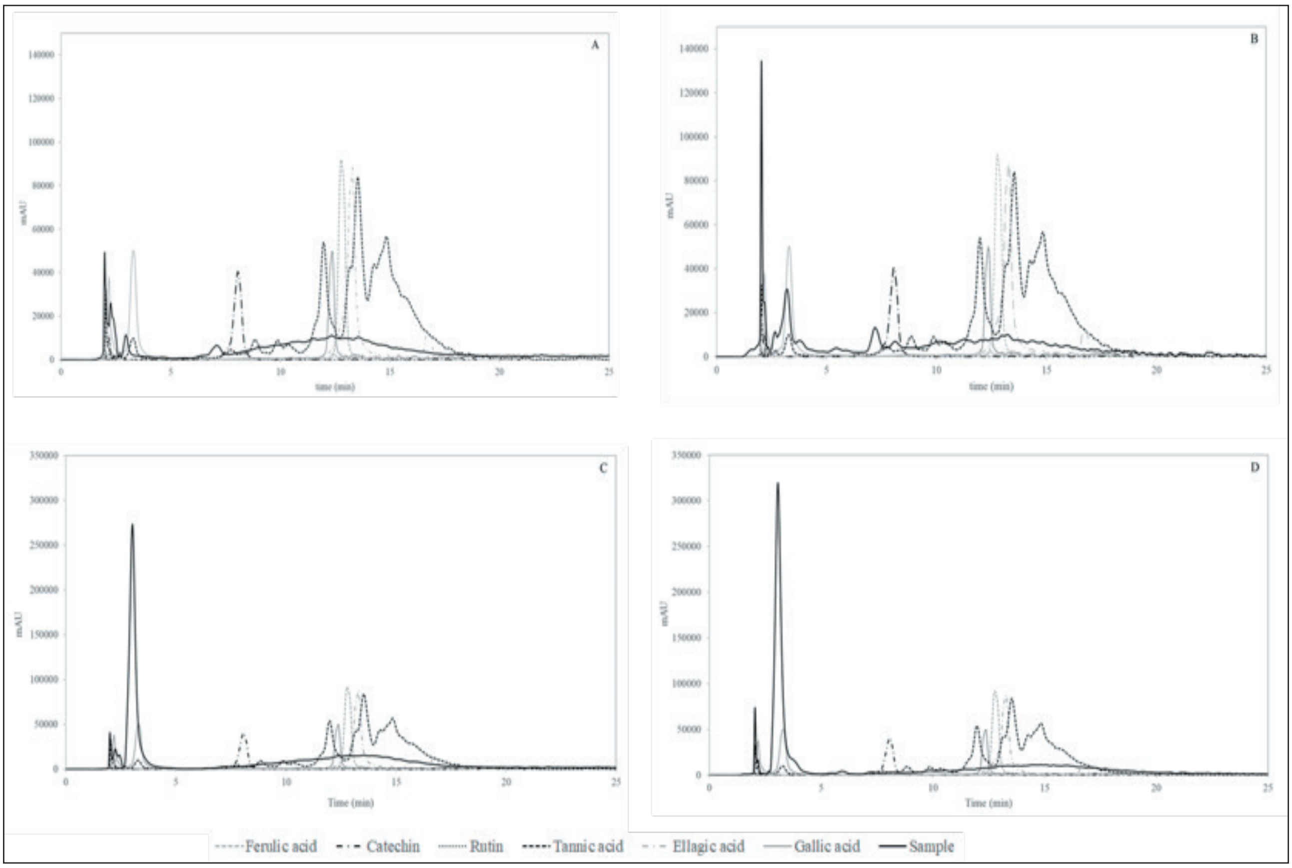
Previous works on the chestnut shell's main structural and functional characteristics (VÁZQUEZ *et al.*, 2008; VÁZQUEZ *et al.*, 2013 and SQUILLACI *et al.*, 2018) demonstrated that chestnut shell tannins are mainly of condensed type. In this nut, the condensed tannins have been measured by the acid/1-butanol method (DE VASCONCELOS *et al.*, 2010), the vanillin/HCl method (HAM; KIM; LIM, 2015; VIUDA-MARTOS *et al.*, 2011) or by the difference in total and hydrolysable tannins (SQUILLACI *et al.*, 2018). In the present work, it was used the acid butanol assay. Acetone:water (80:20) was the most efficient solvent to remove condensed tannins from the chestnut shell compared with water and ethanol:water (80:20). For acetone:water (80:20), the highest concentrations were obtained for US for 1 min and ST for 30 min, indicating that US is a time-efficient method to extract these compounds from chestnut shell. Our results agree with those obtained by DE VASCONCELOS *et al.* (2010), who also observed that 70% aqueous acetone was a suitable solvent for condensed tannins extraction. Extracts rich in condensed and hydrolyzable tannins are potentially more antioxidant (15-30 times more effective at quenching peroxyl radicals) than those of simple monomeric phenolics or Trolox (HAGERMAN *et al.*, 1998). Thus, extracts with ethanol:water (80:20) and acetone:water (80:20) subjected to the US may be rich in hydrolyzable and condensed tannins, respectively, with the potential to be used in food, pharmaceutical and cosmetic industries.

Reversed-phase HPLC analysis

The extracts obtained with ethanol:water (80:20) and acetone:water (80:20) were analyzed by HPLC-PDA to identify some bioactive compounds. The chromatograms obtained are shown in Figure 4.

Both solvents extracted compounds differently. For the ethanol:water (Figures 4A and 4B), the extraction method applied (US and ST) affected the extracted compounds. Even though a first peak was detected in both methodologies, probably related to tannic acids

Figure 4. Chromatograms of the extracts: (A) Ethanol:water subjected to US 5 min; (B) Ethanol:water subjected to stirring for 30 min; (C) Acetone:water subjected to US 5 min; and (D) Acetone:water subjected to stirring for 30 min.



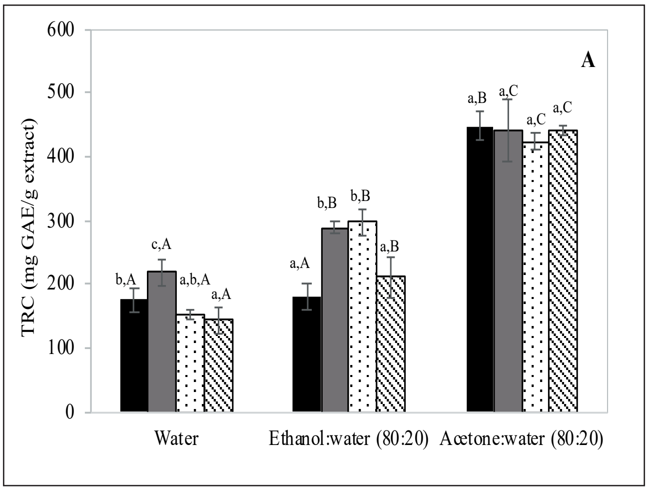
the second peak detected in US for 5 min almost disappeared with ST for 30 min. Moreover, gallic acid and (+)-catechin were observable with this extraction method, contrary to the US for 5 min. Several beneficial effects have been reported for gallic acid, including antioxidant, anti-inflammatory, and antineoplastic properties (KAHKESHANI *et al.*, 2019). Many studies also demonstrate the effects of catechins on the human body and boost their protective power against UV radiation (BAE *et al.*, 2020). There are many examples of catechins' positive antimicrobial, anti-viral, anti-inflammatory, anti-allergenic, and anti-cancer effects (BAE *et al.*, 2020). Furthermore, catechins increase the penetration and absorption of healthy functional foods and bio-cosmetics into the body and the skin, thus improving the utility of these compouds (BAE *et al.*, 2020).

For the acetone:water, the chromatograms obtained were more similar between methodologies, being detected at the beginning of the analysis, higher contents of compounds for ST during 30 min than for US 5 min. However, two compounds could be seen with the latter method, eluting after the first peak, but identifying them was impossible. SORICE *et al.* (2016) also reported a broad and unresolved peak at 275 nm due to the presence of condensed tannins, which may be quite complex, containing up to 12 or more monomeric units of epicatechin, epigallocatechin, and epigallocatechin gallate. This fact can make the analysis of chromatograms difficult. MELISSA NAZZARO *et al.* (2012) and VELLA *et al.* (2019) also detected several phenolic compounds in the chestnut shell, namely, gallic, chlorogenic, ferulic, *p*-coumaric, and ellagic acids, as well as (+)-catechin, (–)-epicatechin, and rutin. In our work, clear detection of rutin was impossible because it coeluted with other compounds, such as tannic acid.

Antioxidant activity

Total reducing capacity The total reducing capacity (TRC) assay was used to estimate the contents of total phenolic compounds present in the chestnut shells. However, this assay is sensitive to other compounds that present reducing properties beyond phenolics. The TRC varied in the following order: acetone:water (80:20) > ethanol:water (80:20) > water. In acetone:water (80:20), the different methods gave similar results. For ethanol:water (80:20), the highest contents were achieved with ST for 30 min and US for 5 min.

Figure 5. (A) TRC content.

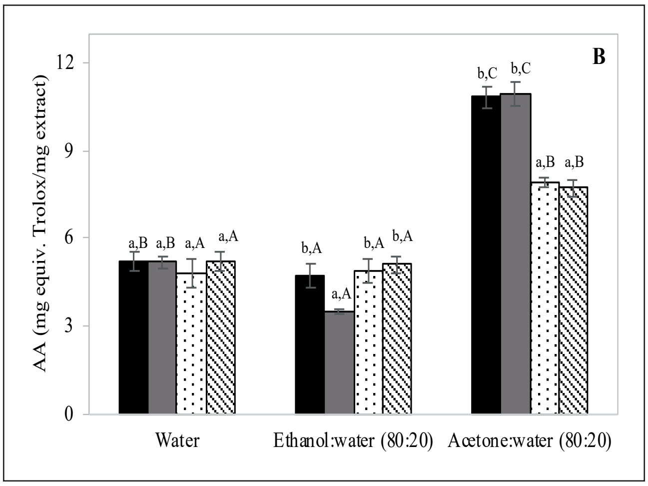


Different lowercase letters mean significant differences between treatments for a given solvent ($p<0.05$) and different uppercase letters mean significant differences between solvents for a given treatment.

Our results agree with those of MELISSA NAZZARO *et al.* (2012), who also obtained larger amounts of total phenols with methanol than with water and DE VASCONCELOS *et al.* (2010), who stated that the efficiency varied in the following order: 70% acetone > 70% methanol >70% ethanol > water. RODRIGUES *et al.* (2015) obtained TRC values between 143 and 797 mg GAE/g db for hydro-alcoholic shell extracts prepared with ethanol:water (1:1) at 50°C for 30 min. These values are higher than those obtained in this work for ethanol:water (80:20), which varied between 2.90 to 4.75 mg GAE/g db, indicating that applying higher temperatures may increase the extraction of the compounds. FERNÁNDEZ-AGULLÓ; FREIRE; GONZÁLEZ-ÁLVAREZ (2015) stated that high temperatures could weaken the plant tissues favouring the release of the phenolic compounds to the solvent.

DPPH radical-scavenging activity The best DPPH radical-scavenging activities were obtained for acetone:water (80:20), when applying the US for 1 and 5 min. These extraction methods presented higher extraction values than the ST method for 30 and 60 min. These results agree with the highest contents of condensed tannins and flavonoids (known by their antioxidant properties) determined in the acetone:water extracts.

Figure 5. (B) Antioxidant activity.

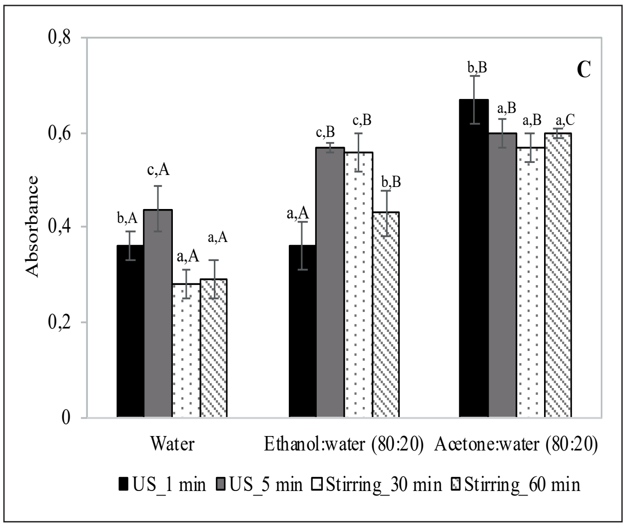


Different lowercase letters mean significant differences between treatments for a given solvent ($p<0.05$) and different uppercase letters mean significant differences between solvents for a given treatment.

The results obtained for water and ethanol:water (80:20) were similar, except for the ethanol:water (80:20) with the US for 5 min that presented the lowest DPPH radical-scavenging activity. With water, it was observed that the method applied did not affect the extraction of compounds responsible for this antioxidant activity, and the US were able to accelerate the extraction process.

Reducing Power The extracts of acetone:water (80:20) had higher reducing power values than the samples extracted with ethanol:water (80:20) and water. For acetone:water (80:20), no significant differences ($p>0.05$) were observed between methods; however, the highest percentage was obtained with the US for 1 min.

Figure 5. (C) Reducing power determined in dry extracts obtained from chestnut shells after applying different solvents and extraction methods (US – Ultrasounds and Stirring).



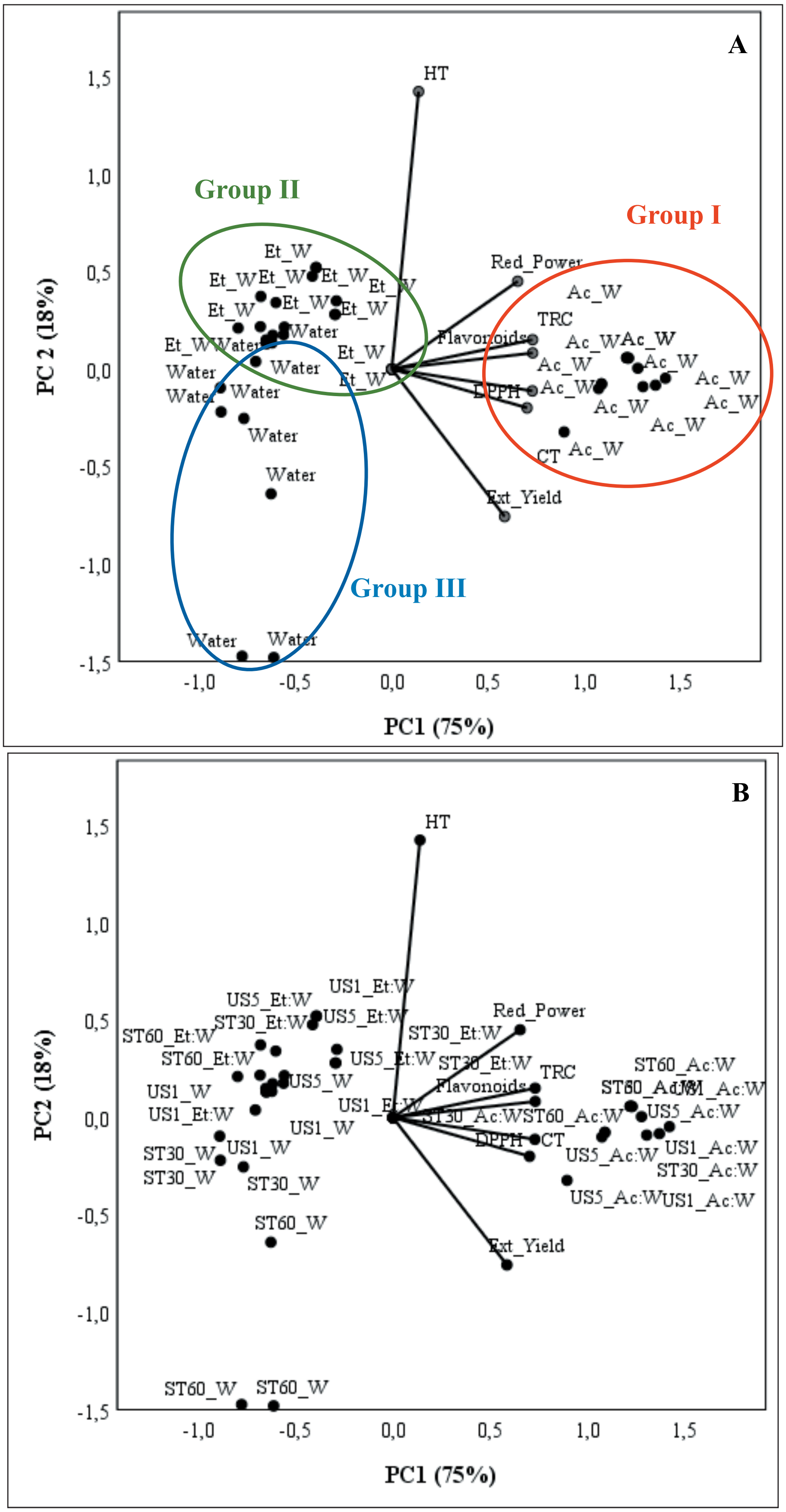
Different lowercase letters mean significant differences between treatments for a given solvent ($p<0.05$) and different uppercase letters mean significant differences between solvents for a given treatment.

For ethanol:water (80:20), the methods of US for 5 min and ST for 30 min originated extracts with higher reducing powers than ST for 60 min and US for 1 min. For water, the best results were observed for US followed by the ST method.

Discrimination of the extracts based on the chemical and antioxidant properties

To summarise the data obtained in the chemical and antioxidant properties of the chestnut's extracts prepared in this work, a PCA was performed (Figure 6). 93% of the total variance of the data could be explained using two principal factors (PC1 = 75%; PC2 = 18%).

Figure 6. Loadings and scores plot resulting from the principal component analysis (PCA) of extraction yield, HT, CT, flavonoids, TRC, DPPH radical scavenging activity, and Reducing Power to separate the samples by the solvent (A), and extraction method + solvent (B).



When considering the solvents (Figure 6A), the samples were naturally gathered into three main groups: Group I is located in the positive region of the first principal factor ("acetone:water extracts"); Group II is located in the negative region of the first principal factor ("ethanol:water extracts"), and Group III is located in the negative region of the second principal factor ("water extracts"). However, some aqueous extracts were close to some ethanol:water extracts. On the contrary, when the treatments were considered (Figure 6B), it was not possible to differentiate them. So, the role of the solvent seems to be higher than the method applied.

The acetone:water solvent was the one that originated the highest extraction yields, as shown in Figure 6A, and the extracts were the richest in flavonoids and condensed tannins and had the most increased antioxidant activity. The ethanol:water solvent allowed the highest extraction of hydrolyzable tannins. In contrast, water was the solvent that originated the extracts with the lowest content of flavonoids and hydrolyzable tannins.

Antimicrobial activity

The antimicrobial activity of phenolic compounds has not yet been totally understood. However, these compounds may inactivate essential enzymes, react with the cell membrane, or alter the function of the genetic material (MARTILLANES *et al.*, 2017).

The antimicrobial activity of the extracts obtained using the different solvents and treatments was assayed against several target Gram-positive (*S. aureus, B. cereus, B. subtilis, E. faecalis*), Gram-negative (*E. coli, K. pneumoniae, P. aeruginosa, E. aerogenes, P. mirabilis*) bacteria and one yeast (*C. albicans*). In general, the extracts obtained by ST did not show any antimicrobial activity independently of the solvent used, while the extracts obtained with the US, exhibited antimicrobial activity, except when ethanol:water (80:20) and US of 5 min were used. Table 1 shows the solvents and the treatment times with the US method that provided antimicrobial activities.

The antimicrobial activity is related to the material's phenolic composition and the type of phenolic compounds present in each extract (FERNÁNDEZ-AGULLÓ *et al.*, 2014). The ethanol:water extracts inhibited the Gram-positive bacteria and the yeast *C. albicans*. On the contrary, these extracts did not show inhibitory activity against Gram-negative bacteria.

Table 1. Antimicrobial activity of chestnut shell extracts (10 mg/mL).

SAMPLES	*Staphylococcus aureus* ATCC 29213	*Enterobacter aerogenes* ATCC 13048	*Klebsiella pneumoniae* ATCC 13883	*Proteus mirabilis* ATCC 14153	*Pseudomonas aeroginosa* ATCC 27853	*Escherichia coli* ATCC 10536	*Bacillus cereus* ATCC 10876	*Bacillus subtilis* ATCC 6633	*Enterococcus faecalis* ATCC 19433	*Candida albicans* spp.
US_1 min Water	–	–	–	+++	–	–	–	–	–	–
US_5 min Water	–	–	–	+++	–	–	–	–	–	–

SAMPLES	*Staphylococcus aureus* ATCC 29213	*Enterobacter aerogenes* ATCC 13048	*Klebsiella pneumoniae* ATCC 13883	*Proteus mirabilis* ATCC 14153	*Pseudomonas aeroginosa* ATCC 27853	*Escherichia coli* ATCC 10536	*Bacillus cereus* ATCC 10876	*Bacillus subtilis* ATCC 6633	*Enterococcus faecalis* ATCC 19433	*Candida albicans* spp.
US_1 min Ethanol:water 80:20 (v/v)	+++	–	–	–	–	–	+++	+++	+++	+++
US_1 min Acetone:water 80:20 (v/v)	–	–	–	+++	–	–	–	–	–	–
US_5 min Acetone:water 80:20 (v/v)	–	–	–	+++	–	–	–	–	–	–

Inhibition zone < 1mm: no antimicrobial activity (-); Inhibition zone 1-3 mm: slight antimicrobial activity (+); Inhibition zone 3-5 mm: moderate antimicrobial activity (++); Inhibition zone 5-9 mm: high antimicrobial activity (+++)

Even though (+)-catechin was detected in ethanol:water extract after stirring for 30 min, and it has been reported that (+)-catechins showed moderate antimicrobial activities and inhibited the growth of *S. aureus* and *P. aeruginosa* (FATTOUCH *et al.*, 2007), no antimicrobial activity was found in these extracts in the present work.

The extracts obtained with acetone:water and water showed inhibitory activity only against *P. mirabilis.* Our results agree with those obtained by RODRIGUES *et al.* (2015). They did not find inhibitory activity against any of the microorganisms tested, namely: Gram-positive bacteria (*S. aureus* and *S. epidermis*), Gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa*), and yeast (*C. albicans*), when they used ethanol: water (1:1) at 50 °C for 30 min (stirring). Furthermore, in our study, also none of our extracts showed inhibitory activity against *E. coli* and *K. pneumoniae*. SILVA *et al.* (2020) studied chestnut by-products extracts at 100 mg/mL (10 times higher than ours) against eight multidrug-resistant bacteria isolated from different sources and two food-borne bacteria. They found that the chestnut shell extracts still had no inhibitory activity against *Salmonella enteritidis* C4220 and *Escherichia coli* C999. These results may be explained by the fact that the cell walls of Gram-negative bacteria are a major barrier for the entry of phenolic compounds into the cell cytoplasm (MARTILLANES *et al.*, 2017). As mentioned by FATTOUCH *et al.* (2007), generally, Gram-(+) bacteria are less resistant to bactericidal polyphenols than Gram-(-). In Gram-(-) bacterial membrane, the existence of negatively charged lipopolysaccharide may explain this effect (IKIGAI *et al.*, 1993), causing repulsion. Given the apparent trend toward the evolution of resistant microbial strains, the results of this work indicate that the chestnut shell extracts could be a beneficial adjuvant agent for treating bacterial infections in addition to antibiotics.

CONCLUSIONS

The present work demonstrates that the chestnut shell showed high contents of bioactive compounds, but their extraction is influenced by the solvent used. Acetone:water (80:20) was the best solvent to obtain high extraction yields, flavonoids and condensed tannins, as well as extracts with antioxidant activity. The ethanol:water extracts of chestnut shells presented the highest content of hydrolyzable tannins. Furthermore, they were able to inhibit the growth of Gram-positive bacteria and the yeast *C. albicans*. The results of this work indicate that the chestnut shell extracts could be a beneficial adjuvant agent, as a preservative due to its antimicrobial properties, for the food industry.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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