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Ultrasonic or Microwave Cascade Treatment of Medicinal Plant Waste

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Abstract: In this study, we present a strategy for valorizing lignocellulosic wastes (licorice root and willow bark) that result from industrial extraction of active principles using water as green solvent and aqueous NaOH solution. The wastes were submitted to severe ultrasound (US) and microwave (MW) treatments. The aim of these treatments was to extract the remaining active principles (using water as a solvent) or to prepare them for cellulose enzymatic hydrolysis to hexoses (performed in an NaOH aqueous solution). The content of glycyrrhizic acid and salicin derivatives in licorice root and willow bark wastes, respectively, were determined. The best results for licorice root were achieved by applying the US treatment for 5 min at 25 °C (26.6 mg glycyrrhizic acid/gDM); while, for willow bark, the best results were achieved by applying the MW treatment for 30 min at 120 °C (19.48 mg salicin/gDM). A degradation study of the targeted compounds was also performed and showed good stability of glycyrrhizic acid and salicin derivatives under US and MW treatments. The soluble lignin concentration prior to enzymatic hydrolysis, as well as the saccharide concentration of the hydrolyzed solution, were determined. As compared with the MW treatment, the US treatment resulted in saccharides concentrations that were 5% and 160% higher for licorice root and willow bark, respectively.

Keywords: lignocellulosic waste valorization; licorice root; glycyrrhizic acid; willow bark; salicin derivatives; ultrasound; microwave; enzymatic hydrolysis

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1. Introduction

Lignocellulosic biomass is the most abundant renewable resource worldwide. The structure of lignocellulose is comprised of cellulose, hemicellulose, and lignin, which are all valuable biomaterial resources [1].

Biomass refers to the biodegradable part of products, waste, and residues from agriculture, forestry, and related industries, as well as industrial and municipal solid wastes. Lignocellulosic biomass is comprised of any renewable organic material from terrestrial plants (energy crops (conventional food crops and non-food energy crops) and forest products) and aquatic plants (algae and seagrass), as well as organic waste and residues from agriculture, pisciculture, silviculture, municipal solid waste, and other wastes [2]. The industrial extraction of natural principles from medicinal plants results in a lignocellulosic residue which is not suitable for animal feed. Thus, this material is considered to be waste and an environmental threat. Currently, there is no proper waste management of such plants (e.g., licorice and white willow); they are burned, buried, or used to obtain biogas [3,4]. Thermochemical processing (e.g., pyrolysis and gasification) is an alternative for the conversion of such lignocellulosic biomass waste into fuels, building blocks, etc. [5,6].

With increasing energy prices and the drive to reduce CO_2 emissions, it is necessary, for economic sustainability, to find new technologies and new process strategies which reduce energy use and maximize valorization of raw materials. The conventional methods used to extract valuable compounds from vegetal materials require relatively high solvent and energy consumptions, long extraction times, or high temperatures that could lead to the targeted compounds degradation. Moreover, regarding the negative impact of organic solvents on the environment, it is necessary to use green solvents, such as water, for the recovery of valuable compounds [7,8]. In recent years, amongst others, efficient extraction processes, such as ultrasound- [9] and microwave-assisted extractions [10], have been developed. The cavitation phenomenon can promote disruption of cellular tissue leading to an increase in mass transfer rate [11]. Considering vegetal material and extraction solvent, during microwave treatment, vegetal material can be heated selectively. Thus, the osmotic pressure that is created inside a vegetal particle ruptures the cell wall, and therefore results in easy release of the bioactive compounds [12].

Licorice (*Glycyrrhiza glabra*), which belongs to the Fabaceae family, originates from the Mediterranean region and Southwest Asia. Licorice root contains a saponin glycoside, i.e., glycyrrhizin (Figure 1a), which is 50 times sweeter than sucrose [13]. Glycyrrhizin, which comprises up to 10–25% of the total active compounds found in licorice root, is considered to be its main bioactive constituent [14]. In addition to saponins, licorice also contains flavonoids, coumarins, and stilbenes [15]. Licorice extract has shown antibacterial and antifungal [16–18], antioxidant [19,20], antiviral [21–23], anti-inflammatory [24,25], antiallergic [26–28], and anticancer [29,30] properties.

White willow (*Salix Alba*) is common throughout Europe, the North African coast, and from central Russia all the way to the Chinese border [31]. The characteristic compounds of willow bark are salicin derivatives, which comprise up to 10% of the total bioactive constituents [32]. In addition to salicin derivatives (Figure 1b), white willow also contains lignans, flavonoids, and tannins [33]. Willow bark has shown anti-inflammatory [34–36], antioxidant [37,38], and antimicrobial [39–41] properties.

The structures of the main active principles in these plants are shown in Figure 1.

In addition to bioactive compounds, licorice root and willow bark contain lignocellulose, which is a resourceful biomaterial part that can be converted to pentoses and hexoses or other valuable compounds after removing the lignin. An ultrasound- or microwave-assisted alkali pretreatment of lignocellulosic biomass to remove lignin can enhance the yield of monosaccharides obtained after the enzymatic hydrolysis of vegetal material [42–44].

In this study, we present a strategy for the valorization of lignocellulosic waste that result from the industrial extraction of active principles in water. The first step of this strategy is the extraction of the residual bioactive compounds from the waste, using US and MW treatments. The second step of valorization of these wastes is the pretreatment with US and MW to render the waste suitable for the enzymatic hydrolysis of cellulose to sugars. Licorice root (*Glycyrrhiza Glabra*) and willow bark (*Salix Alba*) wastes, from the Hofigal SA Company, Bucharest, Romania, were used as feedstock. Thus, the novelty of this study is the use of green and sustainable techniques, firstly, to recover valuable constituents which remain in different lignocellulosic materials after industrial extraction of natural compounds and, secondly, to improve the yield of the enzymatic hydrolysis of cellulose to hexoses.

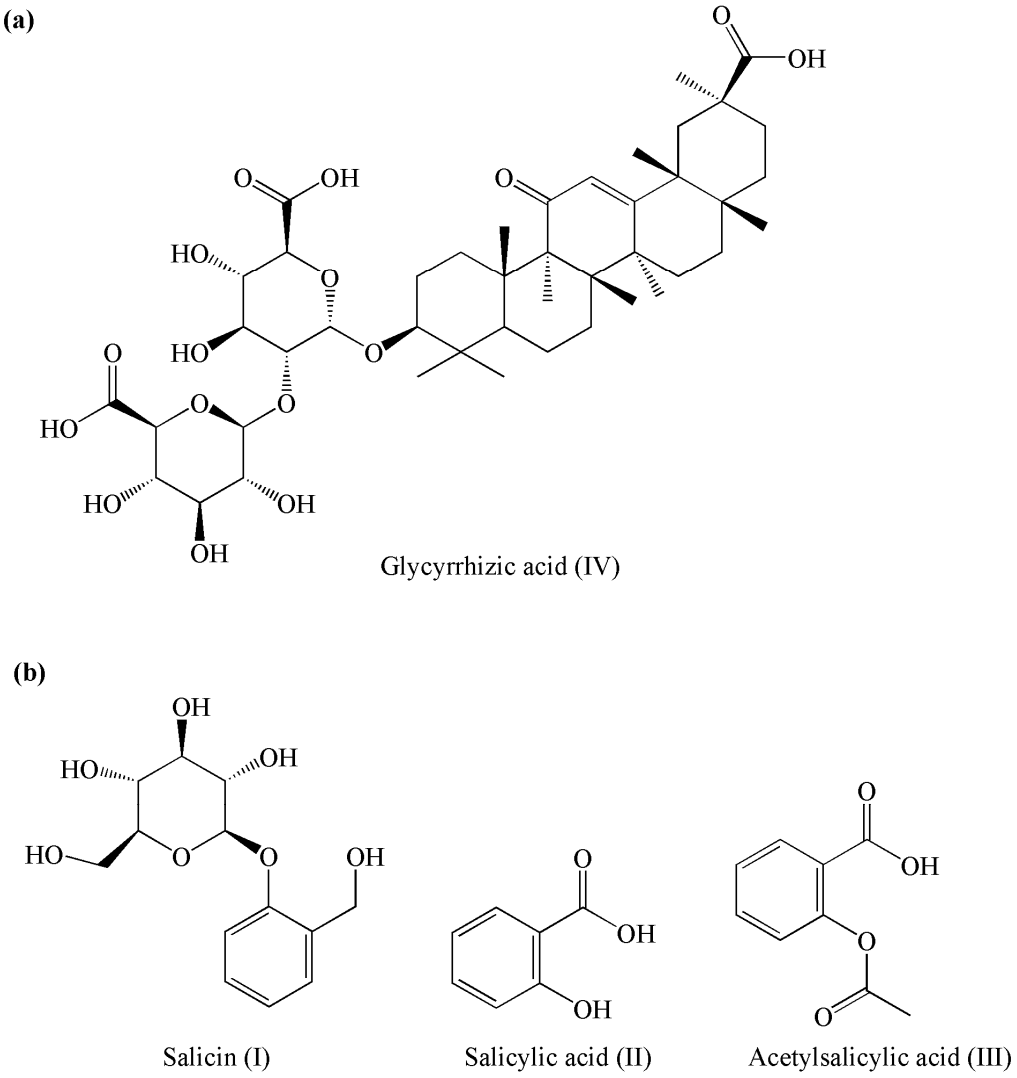

Figure 1. Main active principles of: (**a**) licorice root; (**b**) willow bark.

2. Materials and Methods

2.1. Materials

The licorice root and willow bark waste materials, used in this study, resulted from the industrial extraction of active principles using a ratio of 10:1 (V:w) water to plant, by boiling the mixture for 4–8 h. Further, the solid was separated and the extract was concentrated by vacuum evaporation for 24–28 h, activities which were performed at the Hofigal SA Company, Bucharest, Romania. The solid fraction (considered waste) was dried at 40–45 °C for 2–3 days using an electric dryer oven with trays. The humidity of the waste, after drying, was 14%. Further, the dried vegetal waste was ground using a hammer mill and sieved to a particle size under 315 µm and between 315 and 500 µm for the US and MW treatments, respectively, (the particle size was chosen based on preliminary tests which were part of the research project [45]). To ensure that the targeted compounds were not sensitive to the US or MW treatments, concentrated extracts that resulted from the industrial extraction of active principles from licorice root and willow bark were used to verify the stability of the bioactive compounds under severe US and MW treatments. Kraft lignin with low sulfur content was purchased from Sigma-Aldrich. Glycyrrhizic acid and salicin standards were purchased from Sigma-Aldrich and PhytoLab, respectively. Celluclast 1.5 L enzyme (enzyme activity of 700 endoglucanase units per gram), produced by Novozymes, was used for the enzymatic hydrolysis.

2.2. US and MW Treatments

US and MW treatments were performed in order to recover the remaining active principles from the waste vegetal material (using water as solvent), and then to prepare the exhaust waste for the enzymatic hydrolysis of cellulose to hexoses and pentoses via lignin extraction in a 0.5 N NaOH solution. All the experiments and analyses were performed in triplicate, and the data are presented as mean ± standard deviation. The parameters used for the US and MW treatments were chosen based on preliminary tests that were part of a research project by [45].

2.2.1. US Treatment Equipment and Procedure

The severe US treatment of raw materials was performed using a dual-frequency reactor (Figure 2) equipped with a batch reactor of 600 mL and a stirring system. The US frequencies were 16 and 20 kHz and the maximum power of the two generators was approximatively 600 W.

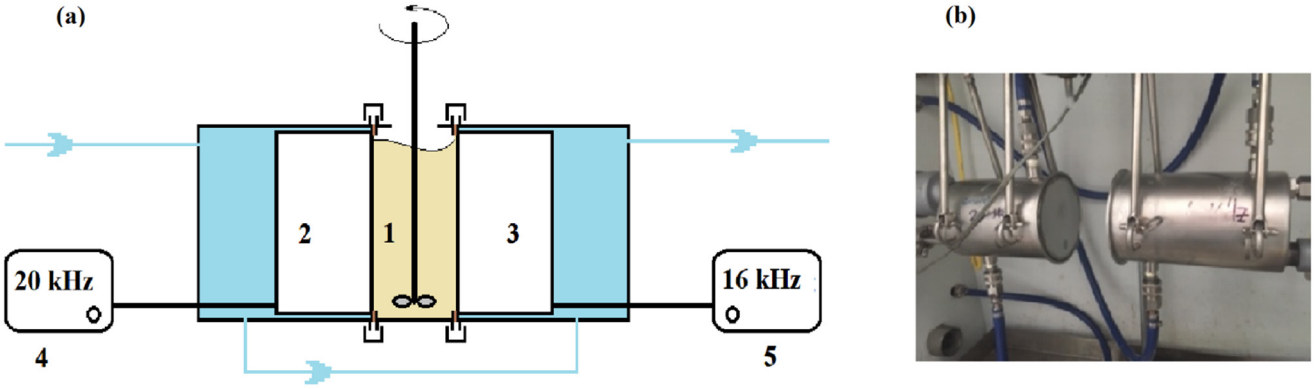

Figure 2. Scheme (**a**) and picture (**b**) of the dual-frequency reactor equipment (Advanced Sonics Processing Systems, USA) for the severe US treatment (1—batch reactor fixed with sanitary flange by the two ultrasound transducers, equipped with stirring system; 2, 3—ultrasound transducers of different frequencies, cooled with water; 4, 5—ultrasound power control systems) [46].

For the licorice root, the US treatment was performed for a 10:1 (V:w) ratio of solvent to plant material. Due to the buoyancy and powdery consistency of the willow bark a 23:1 (V:w) ratio of solvent to plant material was required. The extraction was carried out at a temperature of 25 °C for 5 and 15 min, using an US power of 600 W for each transducer. Since both magnetostrictive transducers are cooled with water during operation, the temperature increase is minimal (2–5 °C).

2.2.2. MW Treatment Equipment and Procedure

The severe MW treatment of raw materials was performed using the Synthwave equipment (Figure 3). This apparatus allows the use of severe conditions: temperatures and pressures up to 300 °C and 200 atm, respectively. Pressurization with an inert gas at such high pressures ensures maintaining the liquid phase of the sample. The equipment also allows the use of a reactor with a volume of maximum 900 mL.

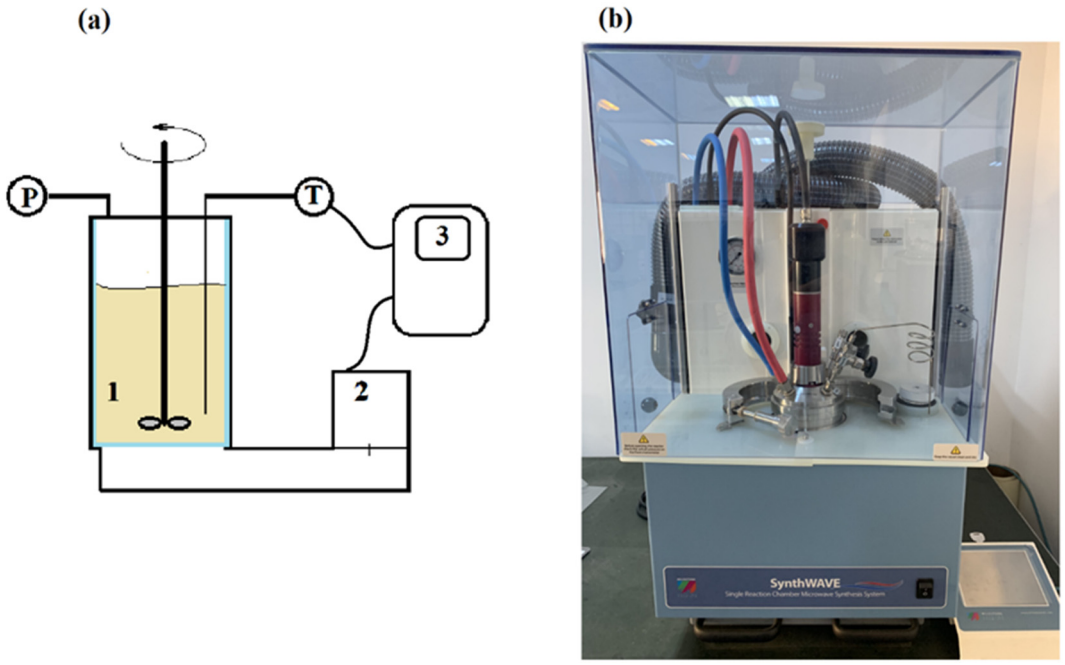

Figure 3. Scheme (**a**) and picture (**b**) of the Synthwave equipment (Milestone, Italy) for the severe MW treatment (1—pressurized reactor equipped with stirring system, temperature, and pressure control systems; 2—microwave generator which transmits the microwaves to the reactor through a waveguide; 3—control system which adjusts the microwave power in accordance with the temperature in the reactor) [47].

The MW treatment was performed at different temperatures (110, 120, and 150 °C) and a pressure of 6–10 atm (higher than vapor pressure of water at reaction temperature). The inert gas used was argon. The experiments were carried out for a 10:1 (V:w) ratio of solvent (water or 0.5 N NaOH solution) to plant material. The extraction time was 30 and 60 min, started after the mixture reached the working temperature. The microwave power required to maintain a constant temperature was in the ranges of 150–200, 200–300, and 375–425 W for 110, 120, and 150 °C, respectively. The frequency was 2.45 GHz, for all the MW treatments.

After the US and MW treatments, the samples were left to settle for one hour before vacuum filtering. The filtrate was subjected to soluble lignin or active principle content determination. Prior to the enzymatic hydrolysis, the solid material was washed with distilled water up to a neutral pH and dried at 50 °C for 8–16 h using a heating oven.

The strategy for the US and MW treatments is shown, as a logic diagram, in Figure 4.

2.2.3. Stability Studies of the Targeted Compounds

The concentrated extracts from the Hofigal SA Company were diluted five times with distilled water and further subjected to US and MW treatments in the same conditions as those for the vegetal material waste, in order to establish the structural stability of the active principles under US and MW irradiation. Then, the treated extracts were analyzed by HPLC to determine the stability or degradation of the glycyrrhizic acid and salicin derivatives.

2.2.4. Delignification of the Waste Materials

Lignin from the lignocellulosic waste materials was extracted to enrich the waste in cellulose in order to be susceptible to hydrolysis to sugars. The US and MW treatments were performed using a solution of 0.5 N NaOH in water.

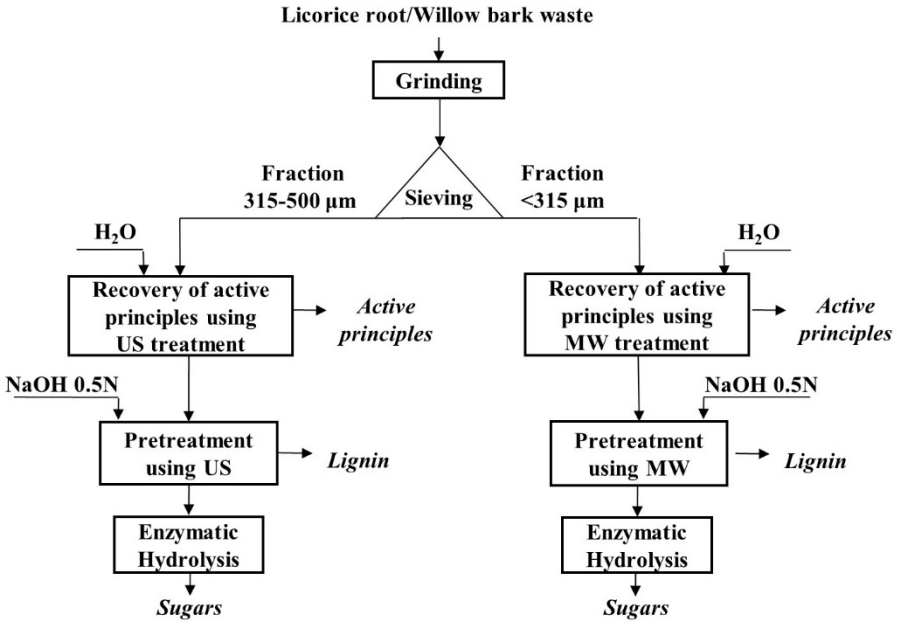

Figure 4. Logic diagram of the industrial lignocellulosic waste valorization using the US and MW treatments.

2.3. Enzymatic Hydrolysis Procedure

Enzymatic hydrolysis requires a pH value of 5 [48]. Therefore, the treated lignocellulosic material for delignification was mixed with a buffer solution containing citric acid and sodium phosphate dihydrate to maintain a constant pH. The experiments were carried out in Erlenmeyer vessels with a 25:1 (V:w) ratio of buffer solution to plant material. Celluclast 1.5 L enzyme (0.7 mL per gram of substrate) was added into each vessel (the enzyme dosage was chosen based on preliminary tests that were part of a research project by [45]). The mixtures were stirred at 120 rpm for 48 h using a reciprocating shaker at a temperature of 50 °C. During the reaction, samples were taken at 24, 48, and 72 h intervals, in separate vials, and quickly immersed in boiling water to deactivate the enzyme. The reaction mixtures were centrifuged at 3500 rpm for 10 min, and the supernatants were further analyzed to determine the saccharide concentrations.

2.4. Analyses

2.4.1. Determination of the Soluble Lignin Content

The soluble lignin content was evaluated according to the Technical Report NREL/TP-510-42618 with minor modifications [49]. The soluble lignin concentration was quantified as milligrams of lignin per 1 g of dry matter (mg lignin/g DM) using a standard curve corresponding to 7–200 mg/L of Kraft lignin (with low sulfur content) solution. The absorbance of the diluted extracts was measured at 320 nm using a Shimadzu UV mini-1240 UV/Visible Scanning Spectrophotometer, 115 VAC.

2.4.2. Determination of Saccharides Concentration

The concentration of sugars (glucose, xylose, arabinose, etc.) that resulted after the enzymatic hydrolysis of lignocellulosic materials was determined by the 3,5-dinitrosalicylic acid method [50,51]. The absorbance was measured at 575 nm using a Jasco V-550 UV/Vis Spectrophotometer. The reducing sugars of the samples were quantified as milligrams of glucose equivalents per 1 g of dry matter (mg GE/g DM) using a standard curve corresponding to 0.24–2 g/L of glucose solution.

2.4.3. Determination of Glycyrrhizic Acid Content

The glycyrrhizic acid content was determined according to the European Pharmacopoeia assay, i.e., monographs on herbal drugs and herbal drug preparations, licorice root, and licorice ethanolic liquid extract, standardized [52]. Thus, approximatively 2.5 g of sample was evaporated using a water bath until 1 g of suspension remained. Further, the sample was mixed with a solution containing one part ultrapure water and four parts methanol and submitted to sonication for 2 min. The mixture was centrifuged at 7800 rpm for 10 min and filtrated on a 0.2 µm polypropylene membrane (PP) filter. The resulting solution was used as the test solution. For the reference solution, 0.1 g of pure glycyrrhizic acid was dissolved in an 8 g/L solution of ammonia reagent and diluted to 100 mL with the same solvent. The glycyrrhizic acid was identified by correlating its retention time of the test solution with the reference solution.

The analyses were conducted using a high-performance liquid chromatography (HPLC) system (Hitachi LaChrom Elite HPLC System produced by Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with the following: a L-2455 diode array detector; a HiCHROM LiChrosorb 100 RP8-10, 10 µm (4.6 × 250 mm) column; a L-2130 pump; an L-2200 autosampler; and an L-2300 column oven. The injected sample volume was 10 µL. The analyses were carried out at a flow rate of 1.5 mL/min using acetonitrile as phase A and 5% acetic acid solution as phase B, under the following program: 30% A and 70% B with a total run time of 35 min. The analytes were detected at 254 nm. The results were quantified as milligrams of glycyrrhizic acid equivalents per 1 g of dry matter (mg/g DM) using a standard curve of 0.05–0.15 mg/mL of glycyrrhizic acid solution.

2.4.4. Determination of the Salicin Derivatives Content

Salicin derivates are the main constituents of willow bark and can be quantified as salicin equivalents. According to the Monography of European Pharmacopoeia [52] and to the Evaluation Report of European Medicines Agency [53], the salicin derivatives content in the bark of different willow species varies from 0.5 to 10%.

Determination of the total content of salicin derivatives was performed according to the adapted assay from the 9th edition of the European Pharmacopoeia [52] as follows: The willow bark sample (1g) was extracted with 8 mL of solution consisting of one part 4.2 g/L NaOH solution and one part methanol. The mixture was stirred at a temperature of 60 °C, at reflux, for 60 min. After cooling down, 0.4 mL of 10 g/L HCl solution was added, and the mixture was centrifuged at 9000 rpm for 5 min. The supernatant was diluted up to 10 mL with a mixture of equal volumes of methanol and ultrapure water. Prior to the HPLC analysis, the samples were filtrated with a 0.2 µm PP filter.

The analyses were conducted using the same HPLC system described in Section 2.4.3. The injected sample volume was 10 µL. The analyses were carried at a flow rate of 1 mL/min using tetrahydrofuran as phase A and 0.005 M phosphoric acid in ultrapure water as phase B, under the following gradient program: 0–8 min 5% phase A and 95% phase B, 9–30 min 10% phase A and 90% phase B, 30–41 min 5% phase A and 95% phase B. The analytes were detected at 270 nm. The results were quantified as milligrams of salicin equivalents per 1 g of dry matter (mg/g DM) using a standard curve of 0.1–0.4 mg/mL salicin solution.

3. Results and Discussion

3.1. US and MW Extractions of Active Principles from the Lignocellulosic Materials

The first step of this study was to verify the US and MW extraction efficiencies of active principles from the two waste vegetal materials, i.e., glycyrrhizic acid from licorice root and salicin derivatives from willow bark. The results are shown in Figure 5.

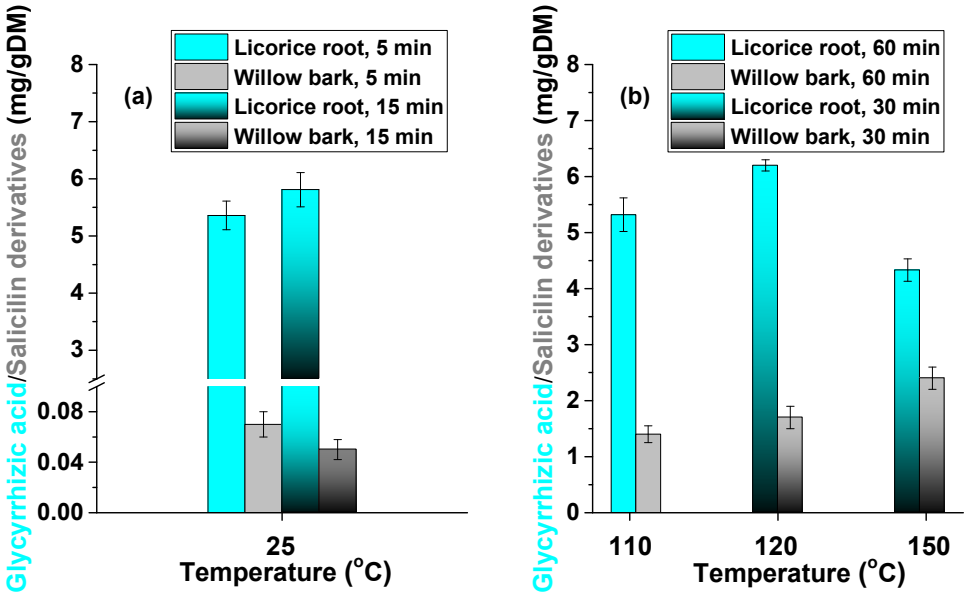

Figure 5. The content of active principles of waste lignocellulosic materials: (**a**) After US treatment; (**b**) after MW treatment.

According to the Romanian Pharmacopeia, the total content of glycyrrhizic acid in licorice root is 33.82 mg/g DM [54]. Experiments performed using US or MW for the extraction from waste materials showed that up to 6.5 mg/g DM of glycyrrhizic acid could be extracted, meaning that the targeted compound was still present in the industrial waste and could be valorized. The higher content of glycyrrhizic acid from licorice root waste was achieved by applying the MW treatment for 30 min at a temperature of 120 °C (Figure 5b); however, a similar value was obtained for the US treatment at only 25 °C for 15 min (Figure 5a).

According to the Romanian Pharmacopeia assay, the salicin derivatives content in unextracted willow bark is 10.94 mg salicin/g DM, while for the industrial waste, it is 0.416 mg salicin/g DM [54].

The analyses of the data presented in Figure 5, indicated that the MW treatment in severe conditions is effective for the extraction of active compounds from both types of waste, while the US treatment is effective only for licorice waste. The latter has a more fragile structure; thus, the milder US treatment is one of the choices when such waste materials need to be valorized. The best results were achieved for the MW extraction at 150 °C, where the salicin derivatives content was approximatively 6 times higher as compared with the Romanian Pharmacopeia assay (Figure 5b). This is due to the selective heating of solid willow bark particles which causes an easier release of the active principles [12,55]. As shown in Figure 5a,b, when the US treatment was applied, the salicin derivatives content from the industrial waste was lower as compared with that when the MW treatment was applied. Thus, the ultrasonic treatment seems to be favorable for the extraction of glycyrrhizic acid as compared with the salicin derivatives.

3.2. Stability of Active Principles during the US and MW Treatments

When a severe treatment, such as US and MW, is applied to natural compounds, it is crucial to ensure that the targeted compounds are not sensitive to these irradiation techniques. Thus, the stability of the active principles from these two plants under US and MW treatments was verified using the extracts that resulted from the industrial unit (by conventional method). The results are shown in Figure 6 for licorice root (Figure 6a) and willow bark (Figure 6b) extracts.

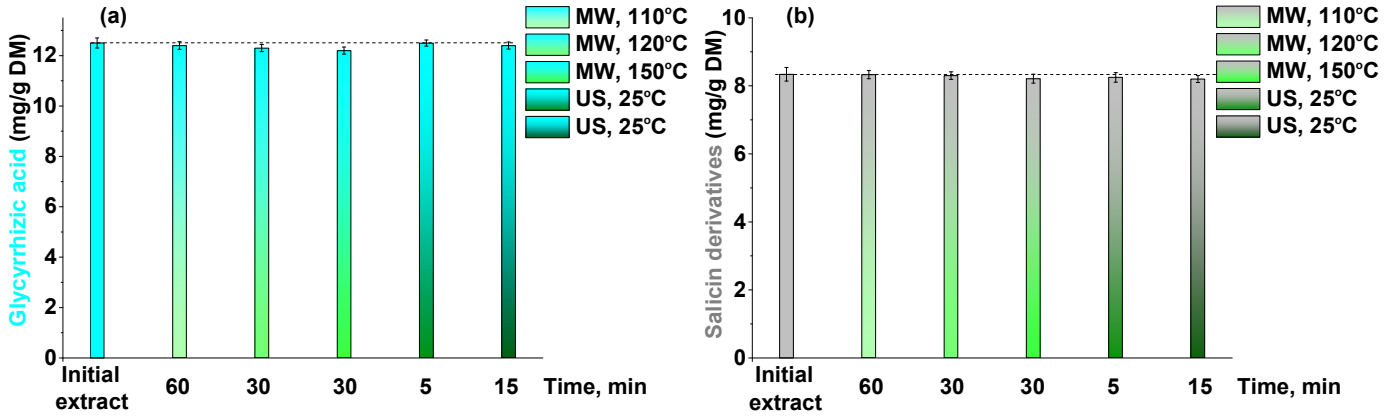

Figure 6. The stability of active principles during severe US and MW treatments: (**a**) Glycyrrhizic acid stability of licorice root extract; (**b**) salicin derivatives stability of willow bark extract.

The extracts resulting from the industrial unit have 12.5 mg/g DM of glycyrrhizic acid and 8.34 mg/g DM of salicin, from licorice root and willow bark, respectively. These values were taken as a reference for the stability study. As shown in Figure 6a, the MW treatment leads to a slight decrease in glycyrrhizic acid content, while under US treatment the degradation of glycyrrhizic acid does not occur. Regarding the stability study of salicin derivatives, it can be observed in Figure 6b that the willow bark extract does not undergo significant damage to the content of active principles when it is submitted to severe US and MW treatments.

According to these results it can be concluded that the US and MW treatments are safe enough to be considered as useful techniques for the recovery of active biocomponents from waste materials ensued from licorice and willow industrial processing.

3.3. Delignification and Enzymatic Hydrolysis of Licorice Root and Willow Bark

Another strategy for the valorization of lignocellulosic waste (which was already processed to extract the active principles) is delignification followed by enzymatic hydrolysis of cellulose to further obtain sugars. The US or MW pretreatment efficiency is monitored by the amount of lignin removed and, secondly, by the determination of saccharides that result after enzymatic hydrolysis. The delignification efficiency is shown in Figure 7.

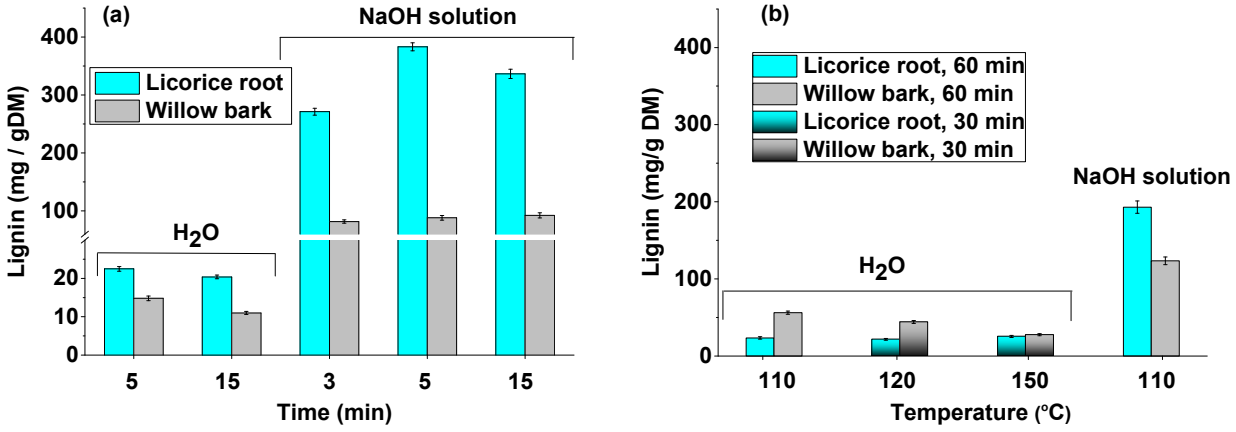

Figure 7. Soluble lignin concentration of the vegetal waste extracts: (**a**) Treated with US; (**b**) treated with MW.

An analysis of the data from Figure 7, shows that the best solvent for delignification is the 0.5 N NaOH solution, for both US (Figure 7a) and MW (Figure 7b) treatments. The soluble lignin concentration after US treatment in the NaOH solution was approximatively 17 and eight times higher than that performed in water, for licorice root and willow bark,

respectively. Considering the US treatment time, the best results were achieved for 5 min (Figure 7a).

The MW treatment in NaOH solution leads to a concentration of soluble lignin that is approximatively eight and two times higher than that carried out in water, for licorice root and willow bark, respectively (Figure 7b).

The results of the enzymatic hydrolysis of delignified lignocellulosic waste are shown in Figure 8. The analysis of the data shows different behaviors for the two biomass wastes. Although the delignification of licorice root waste by the MW treatment is lower as compared with that by the US treatment (Figure 7), it leads to satisfying results for the enzymatic hydrolysis (a saccharide concentration of only 5% lower is achieved for an enzymatic hydrolysis time of 72 h). Regarding the willow bark waste, the US treatment is more efficient than the MW treatment, achieving a saccharide concentration of 160% higher for an enzymatic hydrolysis time of 72 h.

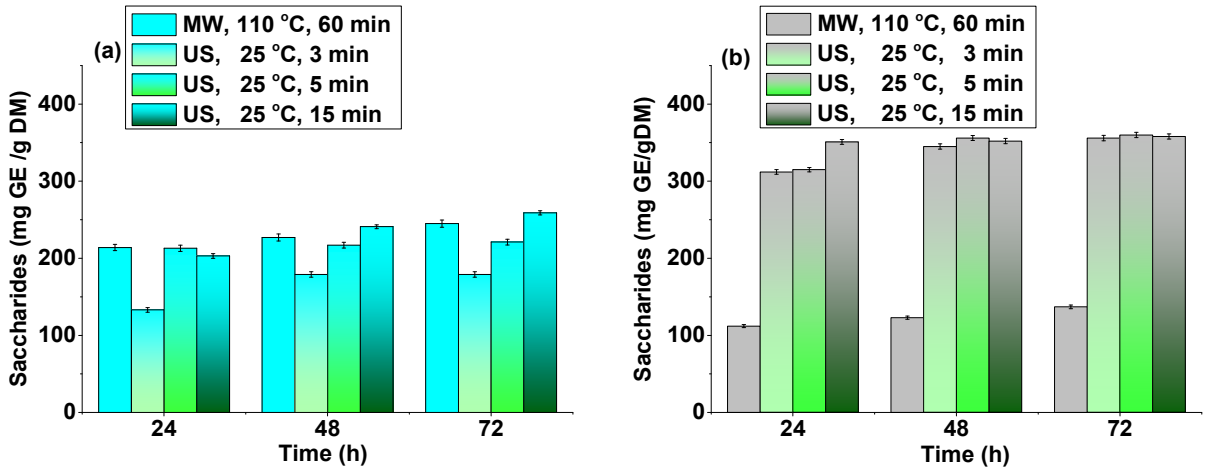

Figure 8. Saccharide concentrations after enzymatic hydrolysis of lignocellulosic waste treated with US and MW in a 0.5 N NaOH solution: (**a**) Licorice root; (**b**) willow bark.

The comparison of these two valorizing methods of the lignocellulosic waste, even in the absence of a strict economic study, leads to the conclusion that the proposed methods can be interconnected, i.e., US treatment is effective for licorice root delignification and willow bark enzymatic hydrolysis; meanwhile, MW treatment is beneficial for extracting both licorice root and willow bark active principles.

3.4. Energy Consideration

Table 1 shows the energy consumption of each type of equipment used to treat the medicinal plant waste. It can be observed that they are comparable. If the use of such installations is considered at a pilot or industrial scale, then, batch reactors can be used for the MW treatment, and due to the shorter residence time, continuous reactors can be used for the US treatment. For such installations, the energy consumption can be significantly lower than those obtained in a laboratory scale.

Table 1. Energy consumption for each equipment used for the medicinal plant waste valorization.

Equipment	Grid Power (kW) *	Time (min)	Energy Consumption (kWh)
US	1.2	5	0.1
		15	0.3
MW	0.35	30	0.175
		60	0.35

* Measured by a wattmeter.

4. Conclusions

Treatments with US or MW were used to valorize two medicinal plant wastes. Extraction of active principles and pretreatment of lignocellulosic substrate for enzymatic hydrolysis were both performed. From the results presented in this study, several important conclusions can be drawn:

The medicinal plant waste still contained significant amounts of active principles; thus, the applied treatments recovered approximately 6.2 mg/DM of glycyrrhizic acid and 2.4 mg/DM of salicin derivatives, which represented 18% and 22% of the initial content of glycyrrhizic acid and salicin derivatives, respectively.

The active principles both showed good stability to support the US- and MW-assisted extraction treatments without significant damages.

Pretreatment with US or MW was very efficient for lignin removal, which allowed the conversion of lignocellulosic biomass into sugars with 250 and 350 mg/g DM of saccharides for licorice and willow wastes, respectively.

The energy consumption of both process types is similar; the US treatment uses a higher power and shorter reaction times, while the MW treatment uses a lower power but requires longer treatment times.

The physical structure of the waste is very different; licorice root waste has a more fragile structure and willow bark waste is more compact and solid. Due to these differences, the optimal treatment is different depending on the type of substrate and the purpose pursued. Thus, for the extraction of the residual active principles from licorice root waste, the US treatment gives results similar to the MW treatment, while the MW treatment is significantly more efficient for willow bark waste. In the case of pretreatment for enzymatic hydrolysis, the removal of lignin from licorice root waste is favored by the US treatment, while, for the removal of lignin from willow bark waste, both types of treatments are similar.

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