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NON-THERMAL PLASMA AS A PRETREATMENT FOR EXTRACTION OF HERBAL TEA BY-PRODUCTS

EKSTRAKCIJA SPOREDNIH PROIZVODA PRERADE BILJNIH ČAJEVA UZ PRETRETMAN GASNOM PLAZMOM

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ABSTRACT

Traditional extraction methods involve the use of polar solvents and/or high temperatures. Both are often energy or time-consuming and require additional purification steps. This compromises the quality of the extracts obtained. Non-thermal plasma pretreatment for extraction is a promising technique that could increase the sustainability of the process while following the principles of hurdle technology. In this work, we investigated the effects of plasma treatment on the aqueous extraction of antioxidant compounds from herbal tea by-products. Stinging nettle, hoary willowherb, and St. John's Wort extracts were treated with plasma at different time intervals. The extracts obtained were analyzed for their antioxidant capacity and total phenolic content. Their potential use as growth media for Ligilactobacillus sp. was also investigated. Hoary willowherb extracts showed the highest total phenolic content and antioxidant activity. While plasma treatment slightly decreased the antioxidant capacity of these extracts, it improved microbial growth. In contrast to hoary willowherb, the antioxidant capacity of stinging nettle extracts' was not affected by plasma treatment. These extracts were also better for bacterial growth. When added to 25% v/v MRS broth, stinging nettle extracts achieved a growth rate similar to that of pure MRS broth (~10^9 CFU/ml), demonstrating their potential for microbial biomass production and food and feed supplementation.

***Keywords:** stinging nettle; hoary willowherb; antioxidant activity; probiotics; waste valorization; gas plasma.*

REZIME

Antioksidansi imaju važnu ulogu u sprečavanju oksidacije masnih kiselina prisutnih u prehrambenim proizvodima, čime značajno produžavaju njihov rok trajanja. Zbog nepovoljnog uticaja pojedinih sintetskih antioksidanasa na zdravlje ljudi, sve je češća upotreba antioksidanasa dobijenih iz prirodnih izvora. Antioksidansi se iz biljnih izvora izoluju ekstrakcijom. Tradicionalne metode ekstrakcije podrazumevaju upotrebu polarnih rastvarača, uz primenu visokih temperatura. Ovi procesi su često energetski nepovoljni, dugo traju i zahtevaju dodatne korake prečišćavanja, kako bi se uklonili toksični rastvarači. Izolovanje biološki aktivnih jedinjenja primenom gasne netermalne plazme kao pretretmana bi moglo da poveća održivost ovog procesa. Osim toga, kombinacijom zelenih rastvarača, poput vode, sa tretmanom plazmom, dobijanje prirodnih antioksidanasa bi bilo u skladu sa tzv. „hardl" tehnologijom i principima cirkularne ekonomije, kojima se teži u prehrambenoj industriji. Predmet ovog rada je ispitivanje mogućnosti dobijanja prirodnih antioksidanasa kombinovanom ekstrakcijom nusproizvoda proizvodnje biljnih čajeva. Vodeni ekstrakti koprive, svilovine i kantariona tretirani su plazmom u različitim vremenskim intervalima. Analizirana je antioksidativna aktivnost dobijenih ekstrakata, kao i ukupni sadržaj fenola. Takođe, ispitivana je i mogućnost upotrebe ovih ekstrakata kao fermentacionog medijuma. Najveća antioksidativna aktivnost i najviši sadržaj ukupnih fenola ostvareni su u ekstraktima svilovine. Ove vrednosti su blago opadale sa povećanjem dužine tretmana plazmom, dok je rast bakterija bio poboljšan. Ekstrakti koprive su posedovali značajno manju antioksidativnu aktivnost i niži sadržaj ukupnih fenola od ekstrakata svilovine. Međutim, ovi ekstrakti su bili pogodniji za gajenje bakterija. Dodavanjem 25% MRS bujona u ekstrakte koprive, postignut je približno isti rast Ligilactobacillus salivarius kao u čistom MRS bujonu.

***Ključne reči:** kopriva, svilovina, antioksidativna aktivnost, probiotici, valorizacija otpada, gasna plazma.*

INTRODUCTION

Antioxidants have an important role in food technology, pharmacy, cosmetology, and medicine. They act as scavengers of free radicals that can induce cell damage. Cell damage leads to the development of various disorders, such as cardiovascular diseases, damage of nucleic acids, and other deteriorative processes (Yanishlieva-Maslarova & Heinonen, 2001). This can be prevented either by scavenging formed free radicals, inhibiting their formation, or promoting their decomposition (Young & Woodside, 2001). For this purpose, different types of antioxidants are added. According to the origin, they can be categorized into two major groups, natural and synthetic antioxidants. Synthetic antioxidants had wider use in the past due to their consistent quality, availability, lower price, and higher efficiency (Yanishlieva-Maslarova & Heinonen, 2001). The most frequently used synthetic antioxidants are butylated hydroxyl anisol (BHA), butylated hydroxyl toluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) (Brewer, 2011; Taghvaei & Jafari, 2015). Recently, there has been a growing awareness of the importance of a healthier lifestyle and a clean diet. Recent reports on toxicity and negative effects of synthetic antioxidants on human health are shifting research interest but also consumers' attention to plant-originating antioxidants (Liu & Mabury, 2020; Viana da Silva et al., 2022).

The main sources of antioxidants among plants are fruits (berries, grapes, pomegranate, date, etc.), vegetables (broccoli, potato, pumpkin, beetroot, etc.), legumes, whole grains, green and black tea, coffee, herbs and spices (rosemary, oregano, cinnamon, sage, thyme, mint, ginger, clove, etc.), wine, and beer (Akbarirad et al., 2016; Shah et al., 2014). Since those resources are used directly in human nutrition, alternative sources of antioxidants for food product supplementing would be preferable. Herbal tea by-products could be used as a source of compounds possessing antioxidant activity. According to the Regulations on the quality of tea, herbal tea, and their products for the Republic of Serbia, only 15% of twigs and other plant parts and 15% of pulverized parts with particles smaller than 0.75 µm are allowed for first-order quality herbal tea (Pravilnik o kvalitetu čaja, biljnog čaja i njihovih proizvoda, 2012). Considering numerous tea producers across the Republic of Serbia, huge amounts of herbal residues are being generated every year. Those residues are usually disposed of by landfilling or are seldom used as fertilizer. Besides overcoming the ecological problem of their accumulation, herbal tea by-products could provide both natural antioxidants and preparations possessing other benefits for human health.

Bioactive compounds present in herbs may be removed as extracts, essential oils, or resins. Different types of solvents could be used for extraction, such as water, or aqueous mixtures of polar solvents (ethanol, methanol, acetone, acetonitrile) (Oguz et al., 2019). The polar solvent-based extraction negatively impacts the environment and often requires high temperatures and a prolonged extraction period, worsening the quality of obtained extracts. Hence, switching to sustainable green extraction techniques is recommended. The first step is the usage of green solvents, such as water or ethanol, and lower process temperature. To successfully extract bioactive substances from plants in these mild conditions, extraction should be combined with some physical or physicochemical process. Non-thermal plasma technology is a novel approach based on the generation of highly reactive species, both oxygen and nitrogen (Đukić-Vuković et al., 2017). Radicals formed during this treatment have low selectivity and are the perfect precursor to the conversion of various organic compounds through different pathways (Grbić et al., 2023). Moreover, these radicals show antimicrobial activity. By combining the suitable process parameters, such as feed gas, plasma configuration, process temperature, treatment duration, and media composition, non-thermal plasma treatment could be implemented as a green treatment in food technology without significant deterioration in the quality of food products (Muhammad et al., 2018).

Medicinal plants are a good source of different bioactive compounds that have health-promoting properties. The proper choice of the medicinal plant along with extraction conditions could provide various preparations and formulations with combined beneficial effects, e.g., antioxidant, antimicrobial, antiviral, analgesic, anti-inflammatory, anti-ulcer, etc. Plants used in this study were stinging nettle (*Urtica dioica* L.), hoary willowherb (*Epilobium parviflorum* L.), and St. John's Wort (*Hypericum perforatum* L.). Stinging nettle has been implemented in the treatment of anemia, gout, eczema, and urinary, bladder, and kidney problems thanks to its biological activity (Đurović et al., 2020). It was proven that stinging nettle extracts possess natriuretic, diuretic, and hypotensive effects (Tahri et al., 2000). Hoary willowherb is mainly used for the treatment of prostatic disorders, especially benign prostatic hyperplasia. There are also literature reports about its antimicrobial and anti-inflammatory properties, besides its antioxidant activity (Hevesi et al., 2009). St. John's Wort extracts are primarily used as anti-depressants. They also showed antiviral, antibacterial, and anticancer activity, and are used in the treatment of gastrointestinal disorders, promoting wound healing, and preventing the development of Alzheimer's disease depending on the extract (Muzykiewicz et al., 2019).

This work investigated the possibility of extracting antioxidant compounds from by-products of herbal tea production. Aqueous extracts of stinging nettle, hoary willowherb, and St. John's Wort were treated with a plasma needle for 5 and 10 minutes, respectively, to study the effect of plasma treatment on the antioxidant capacity of the extracts while sterilizing the extracts. Moreover, the extracts obtained were used as a growth medium for *Ligilactobacillus salivarius* to study the growth-promoting properties of these extracts.

MATERIAL AND METHODS

Extract preparation

Herbal tea by-products used in this study were kindly provided by Adonis d.o.o., Sokobanja, Serbia. All medicinal plants were harvested on the slopes of Rtanj Mountain, Serbia. Extracts were prepared by mixing 1.5 g of stinging nettle, hoary willowherb, and St. John's Wort with 40 mL of water. These samples were subjected to a non-thermal plasma needle for 5 and 10 minutes, while control samples were not treated. The plasma needle used for this treatment is custom-made, and it was previously described (Grbić et al., 2022, 2023; Zaplotnik et al., 2014). The distance between the tip of the plasma needle and samples was 1.5 cm, while Argon was used as a feed gas, with a 0.5 slm flow. After the treatment, these suspensions were kept shaking at 37°C for 24 hours to finish the extraction process. Aqueous extracts were separated from residual biomass by vacuum filtration and subjected to further analysis. All chemicals used in this work were p.a. purity.

Antioxidant activity and total phenolic content determination

The antioxidant activity of stinging nettle, hoary willowherb, and St. John's Wort extracts was determined using spectrophotometric DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging method (Brand-Williams et al., 1995) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) radical-scavenging method (Re et al., 1999). All absorbances were measured using a microplate reader (Epoch Microplate Spectrophotometer, US). Shortly, 50 µL of previously diluted extracts were mixed with 1950 µL of DPPH reagent. The mixtures were mixed well and left in the dark for 30 minutes. The absorbance was read at 517 nm against a blank containing methanol. DPPH scavenging activity was calculated using a standard curve for Trolox and expressed as Trolox equivalent (TE, mmol per mg of dry weight). Similarly, 10 µL of previously diluted extracts were mixed with 2000 µL of freshly prepared ABTS reagent and left in the dark for 5 minutes. The absorbance was read at 734 nm against a blank containing water. ABTS scavenging activity was calculated using a standard curve for Trolox and expressed as Trolox equivalent (TE, mmol per mg of dry weight). The Trolox concentration range used for both standard curves varied from 0.5 to 2.0 mM.

The total phenolic content of extracts was determined using spectrophotometric Folin-Ciocalteu's method (Ramalakshmi et al., 2009). Briefly, 50 µL of previously diluted extracts were mixed with 250 µL of Folin-Ciocalteu's reagent and 3 mL of distilled water. After mixing, 1 mL of 15% (wt/vol) of sodium carbonate was added to the test tubes, the mixtures were mixed again and filled up to 5 mL with distilled water. Test tubes were kept at room temperature in the dark for 2 hours, and the absorbance was measured at 750 nm against a blank containing water instead of extracts. The total phenolic content in the extracts was calculated using a standard curve for gallic acid (in a concentration range from 0.2 to 1.0 mg/mL) and expressed as gallic acid equivalent (GAE, mg per mg of dry weight).

Extract fermentation and bacteria viability determination

To examine the potential prebiotic and growth-promoting properties of extracts obtained from tea production by-products, these extracts were inoculated with *Ligilactobacillus salivarius*, a strain with probiotic potential. Aqueous extracts obtained after vacuum filtration were additionally filtered through 0.22 µm pore filters to provide a sterile medium for bacteria growth. Two types of fermentation mediums were prepared. One consisted of pure extracts, and the other was supplemented with 25% (v/v) MRS broth, a standard nutritional medium for the cultivation of lactobacilli. Both mediums were inoculated with 5% (v/v) *L. salivarius* and incubated at 37°C in static microaerophile conditions. Pure MRS media inoculated with *L. salivarius* was used as a control. At the end of the exponential growth phase (approx. after 18 hours), the number of viable *L. salivarius* colonies was determined using Koch's layered plates method (Koch, 2014).

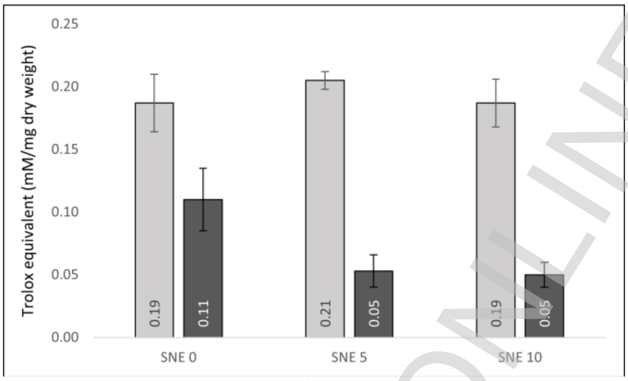
Statistical analysis of data

All measurements were done in biological duplicate. Statistical analysis was performed in OriginPro 9.0 software using a one-way ANOVA analysis of variance with the Tukey test. Differences between the means with probability $p<0.05$ were accepted as statistically significant. The obtained results were presented as mean values and standard deviation as error bars.

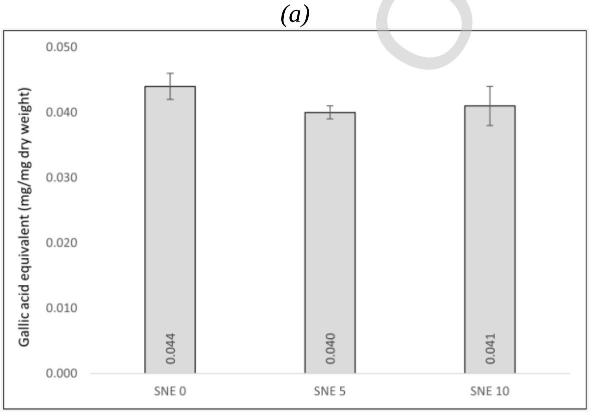
RESULTS AND DISCUSSION

Antioxidant capacity of aqueous herbal extracts

The extracts' antioxidant capacity was estimated using standard DPPH and ABTS methods. The obtained results were significantly different for each plant and should be analyzed separately. The antioxidant capacity and total phenolic content of stinging nettle extracts are presented in Figure 1.



(a)

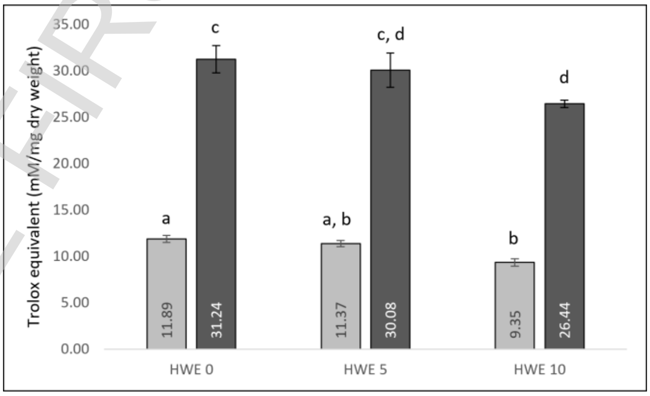


(b)

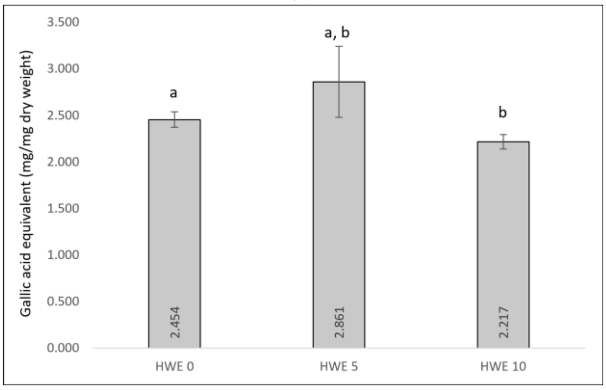
Fig. 1. (a) The antioxidant capacity of stinging nettle extracts (SNE), untreated (0) and plasma treated for 5 or 10 minutes, obtained with the DPPH (light gray) and ABTS (dark gray) method; (b) Total phenolic content of stinging nettle extracts (SNE), untreated (0) and plasma treated for 5 or 10 minutes.

Stinging nettle extracts' antioxidant activity was not affected by plasma treatment. Results showed no significant differences in the total phenolic content between extracts either, proving that plasma could be used for the extraction process without deterioration of the obtained extracts' quality. The total phenolic content of the stinging nettle extracts obtained in this study was around 0.04 mg of gallic acid equivalent (GAE) per mg of dry weight. Literature reports different contents of total phenols in stinging nettle extracts, depending on the extraction method applied. Pogorzelska-Nowicka et al. (2021) applied cold plasma treatment on aqueous herbal extracts. The extraction period was shorter than in our study, but the temperature of the system was around 70°C. Under these conditions, approximately 0.02 mg GAE per mg of dry weight was extracted. López-Hortas et al. (2020) also reported lower phenolic content in aqueous extracts of nettle leaves obtained by microwave hydro-diffusion and gravity at 40°C, up to three magnitudes of order lower than in our extracts. Similarly, during the microwave-assisted extraction, Garofulić et al. (2021) extracted around $5\cdot10^{-3}$ mg GAE per mg of dry weight from nettle leaves. The antioxidant activity of stinging nettle extracts determined by the ABTS method was on average around a hundred times higher than the antioxidant activity reported by López-Hortas et al. (2020).

The highest antioxidant activity and total phenolic content were obtained in hoary willowherb extracts. These results are shown in Figure 2.



(a)

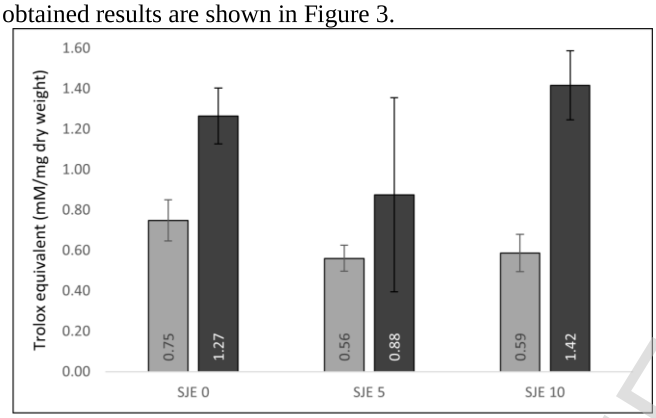


(b)

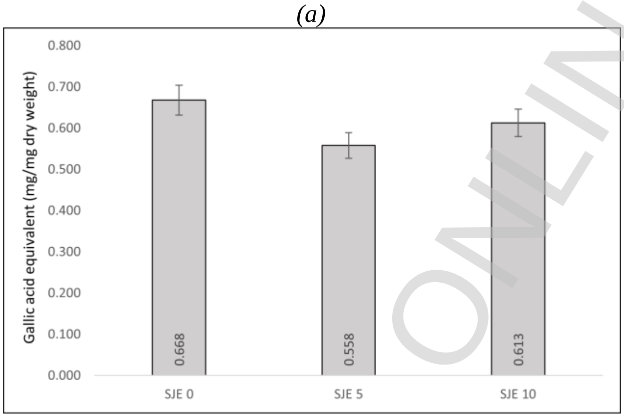
Figure 2. (a) The antioxidant capacity of hoary willowherb extracts (HWE), untreated (0) and plasma treated for 5 or 10 minutes, obtained with the DPPH (light gray) and ABTS (dark gray) method; (b) Total phenolic content of hoary willowherb extracts (HWE), untreated (0) and plasma treated for 5 or 10 minutes. Samples labeled with different letters showed statistically significant differences at the 0.05 level.

The antioxidant activity of hoary willowherb extracts decreased with prolonging the plasma treatment. However, these extracts still had a higher antioxidant activity than those reported in the literature. Vlase et al. (2023) applied ultrasonic-assisted extraction on 30% (v/v) ethanol extracts of hoary willowherb and achieved around 90 mM TE/L extract using the ABTS method. These authors reported the total phenolic content of the same extract of approximately 5 mg GAE/mL extract (Vlase et al., 2023). For comparison, the average antioxidant activity of our hoary willowherb extracts was $4{\cdot}10^5$ mM TE/L, while the total phenolic content was around 35 mg GAE/mL extract on average.

After incubation at 37°C for 24 hours, untreated and 5-minute-long treated St. John's Wort extracts were visibly contaminated by microorganisms already present in the residual biomass. Still, 10-minute-long plasma-treated extracts remained uncontaminated. This proves that prolonged plasma treatment could prevent the spoilage of treated extracts by inactivating microorganisms already present in herbal biomass. This is one of the main applications of plasma in food technology (Matan et al., 2015). St. John's Wort extracts showed slightly higher antioxidant activity and total phenolic content than stinging nettle extracts, but lower than hoary willowherb extracts. The obtained results are shown in Figure 3.



(a)



(b)

Figure 3. (a) The antioxidant capacity of St. John's Wort extracts (SJE), untreated (0) and plasma treated for 5 or 10 minutes, obtained with the DPPH (light gray) and ABTS (dark gray) method; (b) Total phenolic content of St. John's Wort extracts (SJE), untreated (0) and plasma treated for 5 or 10 minutes.

Statistical analysis showed no significant differences between the samples, suggesting that plasma treatment did not have an impact on the antioxidant capacity of St. John's Wort extracts. Again, it was confirmed that plasma treatment could be an effective addition to the extraction process, without worsening the quality of obtained extracts. Despite contamination, these extracts showed higher antioxidant activity and total phenolic content than those reported in the literature. Muzykiewicz et al. (2019) used ultrasound to extract bioactive compounds from aqueous St. John's Wort solutions and obtained 0.003 mg TE per mg raw material by the DPPH method. Pogorzelska-Nowicka et al. (2021) applied previously described cold plasma treatment on St. John's Wort extracts as well. The total phenolic content in these extracts was around 0.06 mg GAE per mg dry weight.

Growth-promoting properties of aqueous herbal extracts

The possibility of using aqueous extracts of herbal tea production residues as a growth medium for *L. salivarius* was examined. The viability of *L. salivarius* at the end of the exponential growth rate is shown in Figure 4. The results are shown only for stinging nettle and hoary willowherb extracts. Due to the visible contamination in St. John's Wort extracts, they were not further subjected to the fermentation step.

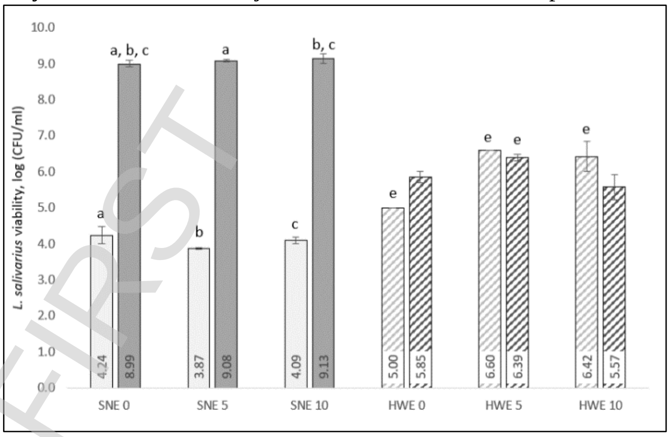


Figure 4. L. salivarius viability in stinging nettle extracts (SNE) and hoary willowherb extracts (HWE, patterned), untreated (0) and plasma treated for 5 or 10 minutes, with (dark gray) and without (light gray) MRS broth supplementation. Samples labeled with different letters showed statistically significant differences at the 0.05 level.

Stinging nettle extracts appeared to be more convenient for bacterial growth than hoary willowherb extracts. When supplemented with 25% (v/v) MRS broth, the nutritional medium generally used for the cultivation of *Ligilactobacillus sp.*, the growth rate of *L. salivarius* was 10^9 CFU/ml, almost the same as in control samples ($5{\cdot}10^9$ CFU/ml). Plasma treatment duration had no impact on the bacteria growth rate. However, it provided a stable pH profile of these extracts during fermentation (data not shown), varying from 5.6 to 6.6, which is an optimal range for *L. salivarius* growth. Although the antimicrobial effect was often reported for different stinging nettle extracts (Garofulić et al., 2021; Zeković et al., 2017), here studied extracts were suitable for the cultivation of bacteria with probiotic potential, especially in combination with MRS.

Hoary willowherb extracts were less suitable for *L. salivarius* growth, reaching a growth rate of around 10^6 CFU/ml. Yet, *L. salivarius* showed slightly better growth in plasma-treated pure extracts than in MRS-supplemented extracts. Prolonged plasma treatment of 10 minutes had no significant effect on bacteria growth. Implementing a 5-minute-long plasma treatment during hoary willowherb extraction could increase bacterial growth in the later fermentation step, while simultaneously increasing total phenolic content with a slight decrease in antioxidant activity. This implies an important link between the extraction of phenolics and microbial growth caused by very short plasma treatment (5 minutes).

This could be an important step up in the production of symbiotic preparations and formulations based on these fermented extracts, possibly having antioxidant and probiotic properties. Depending on the main purpose of the symbiotic preparations, the process could be optimized in a proper way to achieve either higher antioxidant capacity or better bacterial growth-promoting properties.

CONCLUSION

Residues of herbal tea could be used to produce value-added products that have antioxidant and bacterial growth-promoting properties. Under the conditions defined in this study, plasma treatment had a negligible effect on the antioxidant capacity of the extracts. At the same time, prolonged plasma treatment was effective in the inactivation of microorganisms already present in the plant biomass, and prevented spoilage of the treated extracts. In the future, this treatment could be optimized to preserve the antioxidant properties of the extracts. Nevertheless, all three types of extracts obtained from the by-products of herbal tea showed significantly higher antioxidant activity and total phenolic content than the extracts from whole plant parts reported in the literature This could be a great advance in the valorization of herbal waste as it represents a sustainable way to produce extracts with improved quality. However, it has been shown that all the effects observed in this study are substrate-dependent and vary from plant to plant. Therefore, it is necessary to perform independent experiments for each plant considered.

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