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Original Scientific Paper

PLASMA-ASSISTED EXTRACTION OF COMMON NETTLE BY-PRODUCTS FOR LACTIC ACID FERMENTATION

EKSTRAKCIJA SPOREDNIH PROIZVODA PRERADE KOPRIVE POTPOMOGNUTA GASNOM PLAZMOM ZA MLEČNO-KISELINSKU FERMENTACIJU

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ABSTRACT

Nettle (Urtica dioica) is a wild plant rich in flavonoids, carotenoids, phytosterols, and terpenes. It has exceptional pharmaceutical potential due to its anti-inflammatory, antioxidant, and diuretic properties. It is used in food, as tea, or in the form of dry or liquid extracts. To improve the characteristics, bioavailability, and stability of extracts, extractions with environmentally acceptable solvents, green physical treatments, and fermentation with probiotic bacteria are used. Green solvents such as water, ethanol, and biocompatible eutectic liquids are suitable for obtaining extracts following the principles of sustainable development. Innovative physical treatments such as non-thermal plasma treatment can ensure better extraction and microbiological stability, especially of aqueous extracts. Also, bacterial fermentations, such as Ligilactobacillus salivarius, can metabolize the compounds present in the extracts and biotransform glycosides, tannin, and lignan into biologically active forms. In this paper, we examined the growth and antioxidant activity of aqueous extracts of nettle flowers obtained with non-thermal plasma treatment and subsequent fermentation with L. salivarius. A positive combined effect of non-thermal plasma and fermentation on increasing antioxidant activity was observed, especially during shorter treatments with non-thermal plasma. The results of the work indicate the possibility of applying green treatments and fermentation to obtain nettle flower extract products enriched with L. salivarius. Plasma treatment can also be used on the microbiological composition of water extracts, as an important aspect for application in the food and pharmaceutical industry.

***Keywords**: common nettle; antioxidant activity; Ligilactobacillus salivarius; non-termal plasma*

REZIME

Kopriva (Urtica dioica) je samonikla biljka bogata flavonoidima, karotenoidima, fitosterolima, terpenima. Ima izuzetan farmaceutski potencijal zbog svojih antiinflamatornih, antioksidativnih i diuretskih svojstava. Koristi se u ishrani, kao čaj ili u formi suvih ili tečnih ekstrakta. U cilju pobiljšanja karakteristika, bioraspoloživosti i stabilnosti ekstrakata, koriste se ekstrakcije ekološki prihvatljivim rastvaračima, zeleni fizički tretmani i fermentacija probiotičkim bakterijama. Zeleni rastvarači kao što su voda, etanol i biokompatibilni eutektičke tečnosti su pogodni za dobijanje ekstrakata u skladu sa principima održivog razvoja. Inovativni fizički tretmani kao netermalni tretman plazmom mogu obezbediti bolju ekstrakciju i mikrobiološku stabilnost posebno vodenih ekstrakata. Takođe, fermentacije bakterijama, kao što je Ligilactobacillus salivarius, mogu da metabolišu jedinjenja prisutna u ekstraktima i biotransformišu glikozide, tanine i lignane u biološki aktivnije oblike. U radu smo ispitivali rast i antioksidativnu aktivnost vodenih ekstrakata cveta koprive dobijenih uz tretman netermalnom plazmom i potom fermentaciju sa L. salivarius. Uočen je pozitivan kombinovani efekat netermalne plazme i fermentacije na povećanje antioksidativne aktivnosti, posebno pri kraćim tretmanima netermalnom plazmom. Rezultati rada ukazuju na mogućnost primene zelenih tretmana i fermentacije za dobijanje proizvoda ekstrakta cveta koprive obogaćenih sa L. salivarius. Plazma tretman može uticati i na mikrobiološki sastav vodenih ekstrakata, kao važnog aspekta za primenu u prehrambenoj i farmaceutskoj industriji.

***Ključne reči**: kopriva, antioksidativna aktivnost, Ligilactobacillus salivarius, netermalna plazma*

INTRODUCTION

Nettle (*Urtica dioica*), a wild plant native to the cold climate area, contains a wide range of phytochemicals, which implies a wide range of biological activity in humans and animals. It could be found in temperate areas of Europe, Asia, North Africa, and North America at altitudes up to 1800 meters (Bhusal et al., 2022). The phytochemical profile of nettle includes a wide range of compounds such as phenolic acids, flavonoids and their glycosides, fatty acids, pigments, phytosterols, terpenes, tannins, lignans, and alkaloids (Grauso et al., 2020). Variability in chemical compounds can be noticed in seeds, flowers, leaves, stems, and roots. We will highlight it compounds present in nettle flower and seed extracts. Orčić et al. (2014) investigated the chemical profile of methanol extracts of nettle flowers and discovered the persistence of esculetin and scopoletin. Persistence of phenolic acids and their glycosides such as p-hydroxybenzoic, gentisinic, protocatechuic, vanillic, *p*-coumarin, quinine, caffeic, ferulic and 5-O-caffeoylquinic was reported. Also, they reveal the presence of flavonoids and their glycosides, chrysoberyl, kaempferol, isorhamnetin, catechin, kaempferol-3-O-glucoside, quercetin-3-O-glucoside and rutin. Guil-Guerrero et al. (2003) confirmed the presence of fatty acids such as palmitoleic, palmitic, stearic, cis-9,12-linoleic, α-linolenic, gondoic, and erucic in nettle seeds. In nettle flower and leaves extracted with a mixture of water, ethanol and organic solvents presence of hexahydrofarnesyl acetone, neophyte diene, benzyl salicylate, methyl palmitate, ethyl palmitate, phytol and γ-sitosterol was detected by

Iordache et al. (2009). Nettle shows a diversity of biological activities, including anti-inflammatory, antimicrobial, antioxidant, antiprostatic, hepatoprotective, hyperglycemic, hypoglycemic, etc.(Grauso et al., 2020). Yener et al. (2009) confirmed the hepatoprotective properties of nettle seed extract in rats. Hyperglycaemic effects of nettle hydro-alcoholic leaf extract in rats were reported by Namjou et al. (2018). Gohari et al. (2018) observed that treatment with nettle leaf extract significantly reduced blood glucose levels and increased serum insulin levels in diabetic rats. The antioxidative effect of aqueous extract of nettle leaf was proven by Gülçin et al. (2004) Nettle is used in human health and nutrition, as food, as tea, or in the form of dry or liquid extracts.

Bioactive compounds from medicinal plants are commonly used in extracts or some compounds are isolated and purified. Depending on the polarity of extracted compounds extraction solvents need to be chosen. Organic solvents, such as hexane, diethyl ether, acetone, etc., are commonly used for the extraction of non-polar bioactive compounds originating from medicinal plants (Abubakar & Haque, 2020). However, their negative environmental impact is a significant problem. Green solvents such as water, ethanol, natural ionic liquids, and natural eutectic mixtures are sustainable alternatives to organic solvents (Chemat et al., 2019). The benefits of using green solvents are biocompatibility and low environmental footprint, but also, they are less selective to non-polar compounds compared to organic solvents. To achieve better selectivity, green solvents require higher temperatures, longer extraction time, or assistance with some physical treatments. Bioactives of medicinal plants are mostly thermolabile, so low-temperature or non-thermal extraction techniques are preferred. Physical treatments like ultrasound, microwave, pulse electric field, and non-thermal plasma are commonly used for extraction of medicinal plants (Bitwell et al., 2023). These treatments predominantly degrade cell membranes and ensure better diffusion of biologically active compounds from plant materials, but also affect mixing and could induce also chemical changes of the treated material (Krakowska-Sieprawska et al., 2022).

"Plasma is a partially or fully ionized quasi-neutral substance, which includes electrons, ions, neutral particles, molecules in their ground or excited states, free radical species, and electromagnetic radiation" (Piel, 2010). Depending on how the plasma is created, it can be thermal or non-thermal. A variable electric or electromagnetic field produces a partial electric discharge in an inert gas at atmospheric pressure, resulting in non-thermal plasma (Scholtz et al., 2023). The energy of non-thermal plasma is mainly stored in free electrons, while free ions do not make a significant contribution, resulting in a plasma temperature of about 30 to 40°C (Haertel et al., 2014; Vladimir Scholtz et al., 2015). Some of the commonly used non-thermal plasma configurations are dielectric barrier discharge, plasma jet, and corona discharge (Domonkos et al., 2021). Non-thermal plasma is applied in the extraction of bioactive components of plant origin. The mechanism of action of non-thermal plasma is mediated by generation of reactive oxygen and nitrogen species by the interaction of plasma field with components of air (Laroussi, 2020). During the extraction of medicinal plants with non-thermal plasma in aqueous media, can lead fragmentation of biological macromolecules as well as mechanical damage to the cell wall of the extracted plant material, while a high density of free radical species can damage the cell membrane by oxidizing it (Pogorzelska-Nowicka et al., 2021). In extracting plants with non-thermal plasma, degradation of bioactive components can occur, so it is very important to optimize the extraction process itself (Grzegorzewski, 2011; Li et al., 2019). Extracts obtained in this way, if of adequate composition could be also used as growth media for probiotics.

"Live microorganisms that, when dosed in an adequate amount, provide a health benefit to the host" is the generally accepted definition of probiotics (Salminen et al., 2021). *L. salivarius* is bacteria that naturally originate from the human oral cavity and therefore is commonly used as a probiotic for intraoral administration, due to their inherent health benefits (Bogdanović et al., 2024; Nishihara et al., 2014). Many scientific studies have shown the positive impact of *L. salivarius* in the treatment and prevention of dental caries, periodontal disease, and halitosis (Nishihara et al., 2014; Shimauchi et al., 2008). Complex nutritional requirements such as carbohydrates as a carbon source, mineral salts, vitamins, amino acids, and nucleotides are required for lactic acid bacteria (Hébert et al., 2004; Wegkamp et al., 2010). Considering the low content of carbohydrates and amino acids, the application of pure plant extracts as a growth medium for *L. salivarius* could be insufficient to provide all the necessary nutritional requirements for growth. Extracts can be supplemented with MRS (deMann Rogosa Sharp; a standard nutritional medium for the cultivation of *lactobacilli*) to meet all the nutritional needs of this bacteria. Probiotic bacteria are also capable of biotransformation of natural phenolic compounds from natural sources into more bioactive forms (Gaur & Gänzle, 2023).

This study aims to test plasma-activated nettle flower extracts (NFE) as a medium for *L salivarus* growth. We investigated the most efficient dose of MRS as a nettle extract supplement for the best growth of *L. salivarius*. Following the selection of extract-to-MRS ratio, we were investigated the growth of *L. salivarius* and the impact of fermentation on the antioxidant capacity of the supplemented plasma-activated extract. Obtained results could be valuable in producing symbiotic preparations utilizing fermented extracts, potentially featuring both antioxidant and probiotic components.

MATERIAL AND METHODS

Extract preparation

Nettle flower biomass was provided by Adonis d.o.o., Sokobanja, Serbia. Extracts were made by combining 1.5 g of nettle flowers with 40 mL of water and shaking at 37°C for 24 hours. Extracts were separated from biomass by vacuum filtration, sterile filtrated with 22µ filter and subjected to further analysis.

Plasma-activated NFE were subjected to a non-thermal plasma needle for 1, 3, and 5 minutes after the extraction, while control samples were not treated. The custom-made plasma needle utilized in this treatment was previously reported by Grbić et al. (2022). The distance between the plasma needle tip and the samples was 1.5 cm, and Argon was utilized as a feed gas at a flow rate of 0.5 slm.

Antioxidant activity

The antioxidant activity using the DPPH radical scavenging spectrophotometric method (DPPH activity) was described by Brand-Williams et al. (1995). To initiate the reaction, 50 µL of sample was mixed with 1950 µL of DPPH reagent and incubated for 30 minutes in the dark. Absorbance was measured at 517 nm against a blank. DPPH activity was measured using a Trolox standard curve and expressed as Trolox equivalents per gram of biomass.

The antioxidant activity assay with the ABTS radical scavenging spectrophotometric method (ABTS activity) described by Re et al. (1999) was used. To initiate the reaction 10 µL of sample was mixed with 2000 µL of ABTS reagent and incubated for 5 minutes in the dark. Absorbance was measured at 517 nm against a blank. ABTS activity was measured using a Trolox standard curve and expressed as Trolox equivalents per gram of biomass.

Fermentation of the extract and assessment of bacterial viability

To explore the growth-promoting properties and the impact of supplementation, NFE was enriched with varying concentrations (0%, 25%, 50%, and 75%) of MRS and subsequently inoculated with 5% overnight culture of *L. salivarius*. Pure MRS was used as a control sample. All samples were incubated under static microaerophilic conditions at 37°C for 18h. pH of samples was measured before and after fermentation. The after fermentation viability of *L. salivarius* was determined using Koch's layered plates method (Koch, 2014).

After determining the best concentration of MRS for supplementation, fermentation was performed on plasma-activated extracts. Plasma-activated NFE was supplemented with 25% MRS and underwent the same procedure. As a control for the antioxidant activity assays, plasma-activated sterile distilled water was utilized instead of extract, subjected to the same duration of plasma treatment as the extracts. The viability of *L. salivarius* was determined after fermentation, pH and antioxidant activity were measured before and after fermentation.

Statistical analysis of data

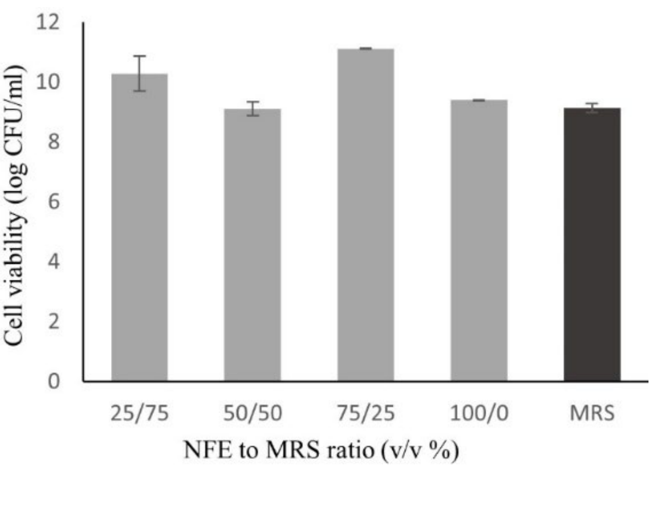
All measurements were taken in biological duplicates. The statistical analysis was done using OriginPro 9.0 software. A one-way ANOVA analysis of variance using Tukey's HSD (Honest Significance Difference) was performed. Statistically significant differences between means were defined as $p < 0.05$. The acquired data are represented as mean values with standard deviations as error bars.

RESULTS AND DISCUSSION

Impact of nettle flower extract supplementation with MRS on *L. salivarius* growth

The nutritional quality of the substrate determines the viability of microorganisms in fermentation. Growth-promoting qualities are essential, particularly for probiotics bacteria, in research aimed at discovering new substrates. We investigated the possible use of NFE supplemented with distinct parts of MRS as a substrate for the growth of *L salivarius*. Figure 1.a) shows *L. salivarius* cell viability in NFE supplemented with MRS, while Figure 1.b) shows the equivalent pH values of the supplemented extracts before and after fermentation.

(a)



(b)

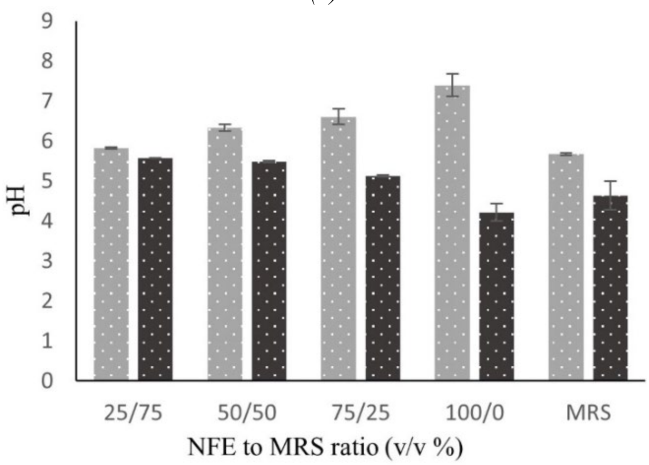


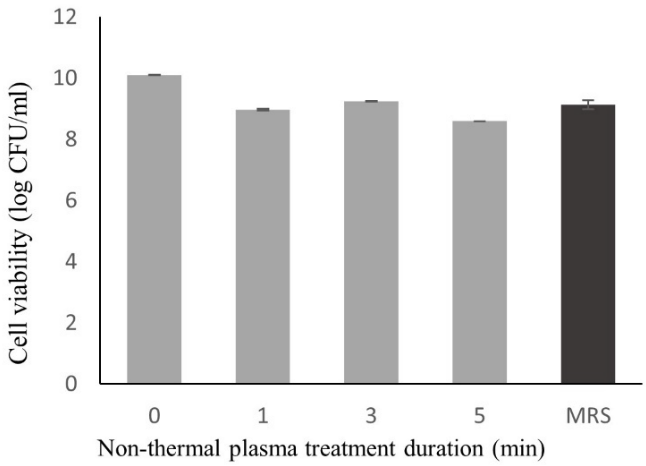
Fig. 1. a) Number of viable L. salivaris cell after fermentation in supplemented NFE; b) pH values of the supplemented extracts before and after fermentation. Symbols: Number of viable of L. salivarus cell in different extract to MRS ratio (v/v %) (light gray), MRS (control) (dark gray), pH cell in different extract to MRS ratio (v/v %) before (light gray patterned) and after fermentation (dark gray patterned)

NFE proved to be a suitable substrate for the growth of *L. salivarius* showed maximum viability at 11 log CFU/ml in the extract supplemented with a mild 25% MRS supplement. The minimum required number of viable cells in probiotic preparations is 9 log CFU/ml (Minelli & Benini, 2008). Pure NFE is considered a low-nutrient medium with almost no presence of reducing sugars, but *L. salivarius* shows almost the same viability as in MRS. Lactic acid bacteria can use compounds such as glycosides, tannins, lignans, etc. as a nutrient to facilitate their growth (Milutinović et al., 2021). Present compounds in NFE are enough for the high viability of *L. salivarius*, but additional supplementation with a mild portion of MRS enhanced microbial growth. The viability of *L. salivarius* is significantly affected by pH, since lactic acid is produced in the fermentation process, therefore the substrate for growth should be capable of maintaining an optimal pH range of 5.5 to 6.5. (König et al., 2009). Since extracts with the addition of 25% MRS meet high vitality and optimal pH, it was decided to examine the vitality and antioxidant activity of plasma-activated extracts with the same proportion of MRS.

Viability of *L. salivarius* and antioxidant activity in plasma-activated extracts

The viability and antioxidant activity were tested in fermented plasma-activated NFE supplemented with 25% MRS. Viability and pH are shown in Figures 2a) and b), respectively. Results of the relative antioxidant activity assessed by DPPH and ABTS assay were presented in Figures 3. a) and b), respectively. To mitigate the influence of MRS supplementation and *L. salivarius* products on antioxidant activity, the presented results were obtained by subtracting the control values (inoculated plasma-activated water with the addition of 25% MRS) from the values of the inoculated plasma-activated extracts and present relative antioxidant activity.

(a)



(b)

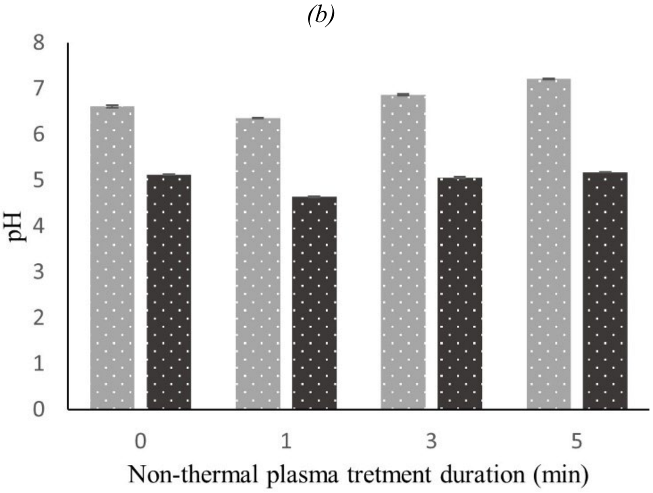
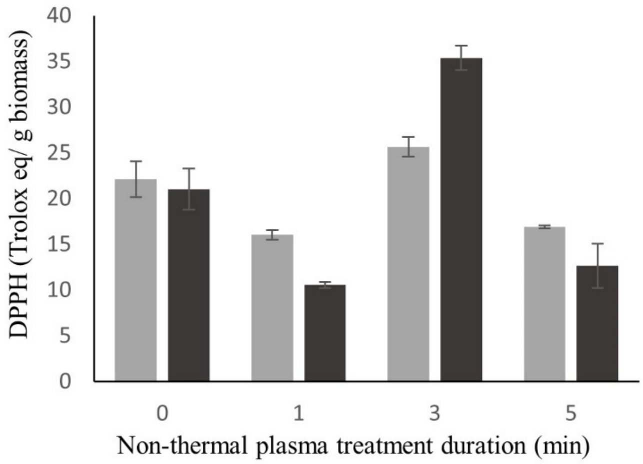


Fig. 2. a) Effect of plasma treatment duration on number of viable L. salivarius cells after fermentation; b) Effect of plasma treatment duration on pH values of fermented plasma-activated NFE. Symbols: Number of viable of L. salivarus cells in supplemented plasma-activated NFE exposed to different treatment times (light gray), MRS (control) (dark gray), pH of supplemented plasma-activated NFE exposed to different treatment times before (light gray patterned) and after fermentation (dark gray patterned)

(a)



(b)

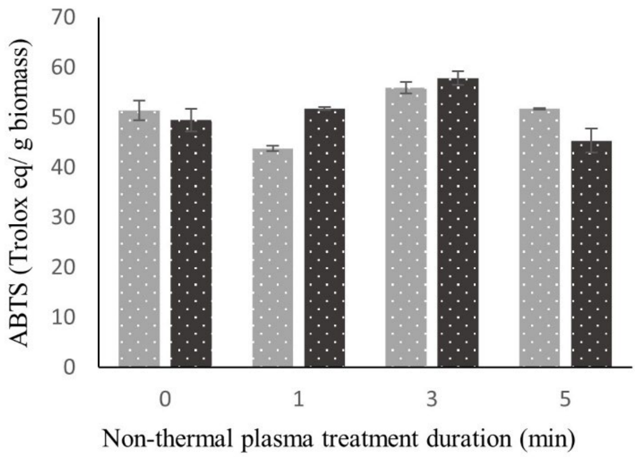


Fig. 3. Effect of plasma treatment duration on relative antioxidant activity by DPPH a) and ABTS b) assay. Symbols: DPPH activity before fermentation (light gray), DPPH activity after fermentation (dark gray), ABTS activity before fermentation (light gray patterned), ABTS activity after fermentation (dark gray patterned)

The viability of *L. salivarius* in plasma-activated NFE supplemented with 25% MRS was preserved and was almost the same viability as in MRS. Non-thermal plasma-activated water, as an agent with high oxidative potential, could be used in disinfection because of its antimicrobial properties (Chiappim et al., 2021). Present free radicals, hydrogen peroxide, and nitric acid, generated in interactions of non-thermal plasma with air or water are mostly responsible for their antimicrobial properties. Antioxidants present in extracts have a protective ability against free radicals generated by non-thermal plasma, but with prolonged plasma treatment protective potential of extracts becomes less effective. The protective effect of the extract is not only reflected in its antioxidant activity but also in higher pH values that buffer the nitric acid or peroxide produced during the plasma treatment and the lactic acid produced during fermentation. The high concentration of calcium (168.77 mg/100g biomass) is probably responsible for the high pH values of NFE (Adhikari et al., 2016).

The highest DPPH activity of 35.39 Trolox eq/ g of biomass is observed in fermented NFE treated with non-thermal plasma for 3 min. The synergistic effect of non-thermal plasma treatment and fermentation is noticeable in the extract treated for 3 min with an increase of 27% in DPPH activity. There is also a noticeable increase in ABTS activity in fermented extract treated for 1min. The difference in DPPH and ABTS activity is probably related to the difference in polarity of detected compounds (Sharopov et al., 2015) and their selectivity towards specific radicals. Carvalho et al. (2017) tested antioxidant DPPH and ABTS activity of extract prepared by maceration of 10g arial parts of common nettle in 200ml 50% aqueous ethanol for 24h. To compare the results with ours, they were converted to Trolox eq/g of biomass. Results revealed ABTS activity of 26 Trolox eq/ g of biomass and DPPH activity of 28.9 Trolox eq/ g of biomass. López-Hortas et al. (2020) report similar results to DPPH activity of best NFE examined in this study and around 6 times lower values in ABTS activity for microwave-extracted nettle leaves. Flórez et al. (2022) optimized extraction of nettle leaves in water, methanol, and ethanol combined with ultrasound-assisted without and with stirring. They reported DPPH radical scavenging activity from 25.9 to 91.1% of the obtained extracts, while our best fermented extract showed inhibition of 56.2% after recalculation to percentage of DPPH inhibition.

CONCLUSION

The study on the growth-promoting qualities of NFE supplemented with different amounts of MRS for the cultivation of *L. salivarius* revealed promising results. NFE, as a low-nutrient medium, showed suitability for robust microbial growth, with optimal viability achieved with 25% MRS supplementation. In non-thermal plasma treated extracts, *L. salivarius* preserved vitality and contributes to the increase in antioxidant activity, showing a synergistic effect of treatment with fermentation. The observed advantages, including the high viability of *L. salivarius* and high content of antioxidant components, emphasize potential of plasma-activated extracts of nettle flowers. Simple preparation using shorter (1min, 3 min), low energy treatments enables valorization of nettle flowers biomass in new ways, beyond more conventional extraction. This study provides valuable insight into the use of nettle extract for microbial growth and increased antioxidant activity through plasma treatment and fermentation, paving the way for applications in the development of symbiotic preparations.

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