Utjecaj ekstrakata nefermentiranog i fermentiranog japanskog dvornika (Fallopia japonica) na stupanj oksidacije u kvascu Saccharomyces cerevisiae

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UNIVERSITY OF RIJEKA

DEPARTMENT OF BIOTECHNOLOGY

Master's degree

"Medicinal chemistry"

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ABSTRACT

The process of oxidation within the human body causes damage to various cellular components. During this process, free radicals are created which cause cellular damage. Antioxidants are compounds that prevent damage within cells by neutralizing the free radicals or by interfering in chain reactions involving free radicals. Despite the reputation of Japanese knotweed (*Fallopia japonica*) as the most invasive species, it has been shown to be a high source of different bioactive compounds with potential antioxidant activity. Lactic acid fermentation can be used to improve the nutritional as well as functional value of substrates including antioxidant activity. This study aimed to compare the impact of non-fermented and fermented Japanese knotweed on oxidation level in yeast *Saccharomyces cerevisiae*. The fermentation of *F. japonica* was carried out by *Lactobacillus plantarum* and evaluated by measuring pH values and *L. plantarum* (LAB) growth. In ethanol extracts prepared from fermented and non-fermented *F. japonica,* total phenolic content using the Folin-Ciocalteu reagent, antioxidant activity by DPPH assay, and antioxidant activity in the cells were measured. The maximum decrease in pH value is observed in the first 24 hours. However, the maximum LAB growth is seen in the first 48 hours. Total phenolic content decreased in 48 hours of fermentation, but DPPH radical scavenging activity increased in the first 48 hours of fermentation. The antioxidant capability of *F. japonica* was further proved with the decrease of oxidation level in cells of yeast *Saccharomyces cerevisiae*. Therefore, the biomass of *F. japonica* has demonstrated its potential as a source of antioxidants.

Keywords: *Fallopia japonica*, lactic acid fermentation, *Lactobacillus plantarum*, total phenolic content, antioxidant activity

SAŽETAK

Proces oksidacije uzrokuje oštećenje različitih staničnih komponenti unutar ljudskog tijela. Tijekom tog procesa stvaraju se slobodno radikali koji uzrokuju oštećenje stanica. Antioksidansi su spojevi koji sprječavaju oštećenja unutar stanica neutraliziranjem slobodnih radikala ili miješanjem u lančane reakcije koje uključuju slobodne radikale. Unatoč reputaciji japanskog dvornika (*Fallopia japonica*) kao najinvazivnije vrste, pokazalo se da je visoki izvor različitih bioaktivnih spojeva s potencijalnim antioksidativnim djelovanjem. Mliječna fermentacija može se koristiti za poboljšanje nutritivne, kao i funkcionalne vrijednosti supstrata, uključujući antioksidativno djelovanje. Cilj ovog istraživanja je usporediti utjecaj nefermentiranog i fermentiranog japanskog dvornika na razinu oksidacije u kvascu *Saccharomyces cerevisiae*. Fermentacija *F. japonica* provedena je pomoću *Lactobacillus plantarum* i određena mjerenjem pH vrijednosti i rasta *L. plantarum* (LAB). U etanolnim ekstraktima pripremljenim iz fermentirane i nefermentirane *F. japonica*, izmjeren je ukupni sadržaj fenola korištenjem Folin-Ciocalteu reagensa, antioksidacijska aktivnost DPPH testom i antioksidativna aktivnost u stanicama. Maksimalno smanjenje pH vrijednosti uočeno je u prvih 24 sata. Međutim, najveći rast LAB-a vidljiv je u prvih 48 sati. Ukupni sadržaj fenola smanjio se u 48 sati fermentacije, ali se aktivnost hvatanja DPPH radikala povećala u prvih 48 sati fermentacije. Antioksidativna sposobnost *F. japonica* dodatno je dokazana smanjenjem razine oksidacije u stanicama kvasca *Saccharomyces cerevisiae*. Stoga je biomasa *F. japonica* pokazala svoj potencijal kao izvor antioksidansa.

KLJUČNE RIJEČI: *Fallopia japonica*, mliječna fermentacija, *Lactobacillus plantarum,* ukupni sadržaj fenola, antioksidativno djelovanje

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1. INTRODUCTION

1.1. Japanese knotweed (*Fallopia japonica*)

Japanese knotweed (Figure 1), also known as *Fallopia japonica, Reynoutria japonica (R. japonica),* or *Polygonum cuspidatum (P. cuspidatum),* is one of the most invasive exotic species in Europe. It is even included in the list of the "100 of the World's Worst Invasive Alien Species" (ISSG) due to its ability to grow in different types of habitats. Originating from Asia and North America, it was first introduced in Europe as an ornament at the end of the 19th century. Japanese knotweeds consist of long branches, thick rhizomes, and large elliptical blade leaves. Rhizomes and stems of *F. japonica* can easily re-sprout and usually are carried by streams, animals, or humans. Although the broad rhizome network of *F. japonica* is almost entirely concentrated in the first meter of the soil, it can extend vertically to 4.5 meters in depth and 20 m sideways from the primary stand. It usually blooms in late summer by developing long white-yellowish panicles in the axils of the leaves. *F. japonica* can be generally found in sunny places, often on the riverside or in pastures. Some of the main reasons for its invasiveness are strong regeneration capacity, high hybridization capability, and abundant resistance to various habitat conditions. Therefore, it is profoundly difficult and costly to remove Japanese knotweed from invaded areas and its management is most of the time not successful. Its strong root system is capable of damaging concrete foundations causing deterioration of buildings, roads, or other architectural sites [1,2]. However, the prevailing approach in the handling of *F. japonica* is chemical control. The main active ingredients that are used for chemical control are glyphosate, synthetic auxins, and acetolactate synthase inhibitors (ALS). Another way to control *F. japonica* is the application of a wide range of herbicides for knotweed control [3]. Moreover, mechanical removal by mowing Japanese knotweed is only a temporary solution since the plant is not suppressed in this way. It only stimulates the formation of new shoots [4]

***Figure 1**: Japanese knotweed* [4]

1.2. Bioactive compounds in *Fallopia japonica*

Japanese knotweed, as an invasive species, poses a threat to native flora and the ecosystem due to its ability to inhibit the growth of other vegetation. The reason behind its vigorous inhibition of other plants is due to the chemical composition of *F. japonica*. Despite the negative impact of Japanese knotweed on its surroundings, it has numerous health benefits. *F. japonica* is considered to have antioxidant, anti-inflammatory, antimicrobial, and anticancer properties among many others. Yet, different parts of Japanese knotweed contain different bioactive compounds. Therefore, different morphological parts of the plant have different applications[1,5].

According to Lachowicz et al.[5], the leaves of *F. japonica* present a greater source of polymeric procyanidins, flavones and flavonols, phenolic acids, oleanolic, and ursolic acids than any other part of the plant. Due to the presence of polyphenolic compounds and triterpenoids, leaves can be used as food additives. Nonetheless, the roots of the plant have more flavan-3-ols, stilbenes such as resveratrol, and other derivates.

1.2.1. Polymeric procyanidins

Polyphenols are the largest group of secondary plant metabolites, and they exhibit various biological activities. Procyanidins are a subset of polyphenols that stem from polyanthocyanidins (a type of condensed tannins). Procyanidins are built from flavon-3-ols (+)-catechin and (-)-epicatechin (Figure 2). They can be classified into A-type and B-type depending on the linkage between monomers and stereo configurations. B-type procyanidins consist of the single bond between C-4 of the B-ring of one (epi)catechin and C-8 or C-6 of the C-ring of another. Besides this bond, A-type has another linkage between the A-ring hydroxyl group and C-2 of the A-ring of another (epi)catechin. The most common B-type procyanidins are B1, B2, B3, and B4, but the most abundant A-types are A1 and A2 which are shown in Figure 3. Procyanidins can be categorized based on their degree of polymerization (DP). Oligomers consist of 2-7 monomeric units, and they can be further polymerized into polymers [6].

Most of the polymeric procyanidins that we consume can be found in cocoa, berries, grapes, pinto beans, and brown sorghum. Polymeric procyanidins have been found to have several health benefits. They have antioxidant properties, helping to protect the cells from free radical damage. Studies suggest that they have anti-cancer properties, especially preventing cancer in the digestive tract. In addition, polymeric procyanidins have been found to prevent diabetes and cardiovascular diseases. In fact, they have an anti-inflammatory effect which is a risk factor inducing cancer and other chronic diseases [7,8].

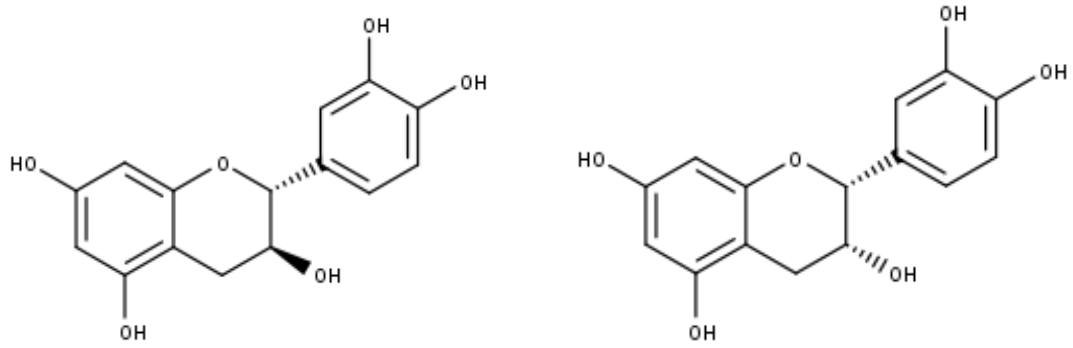

***Figure 2**: Chemical structure of (+)-catechin and (-)-epicatechin*

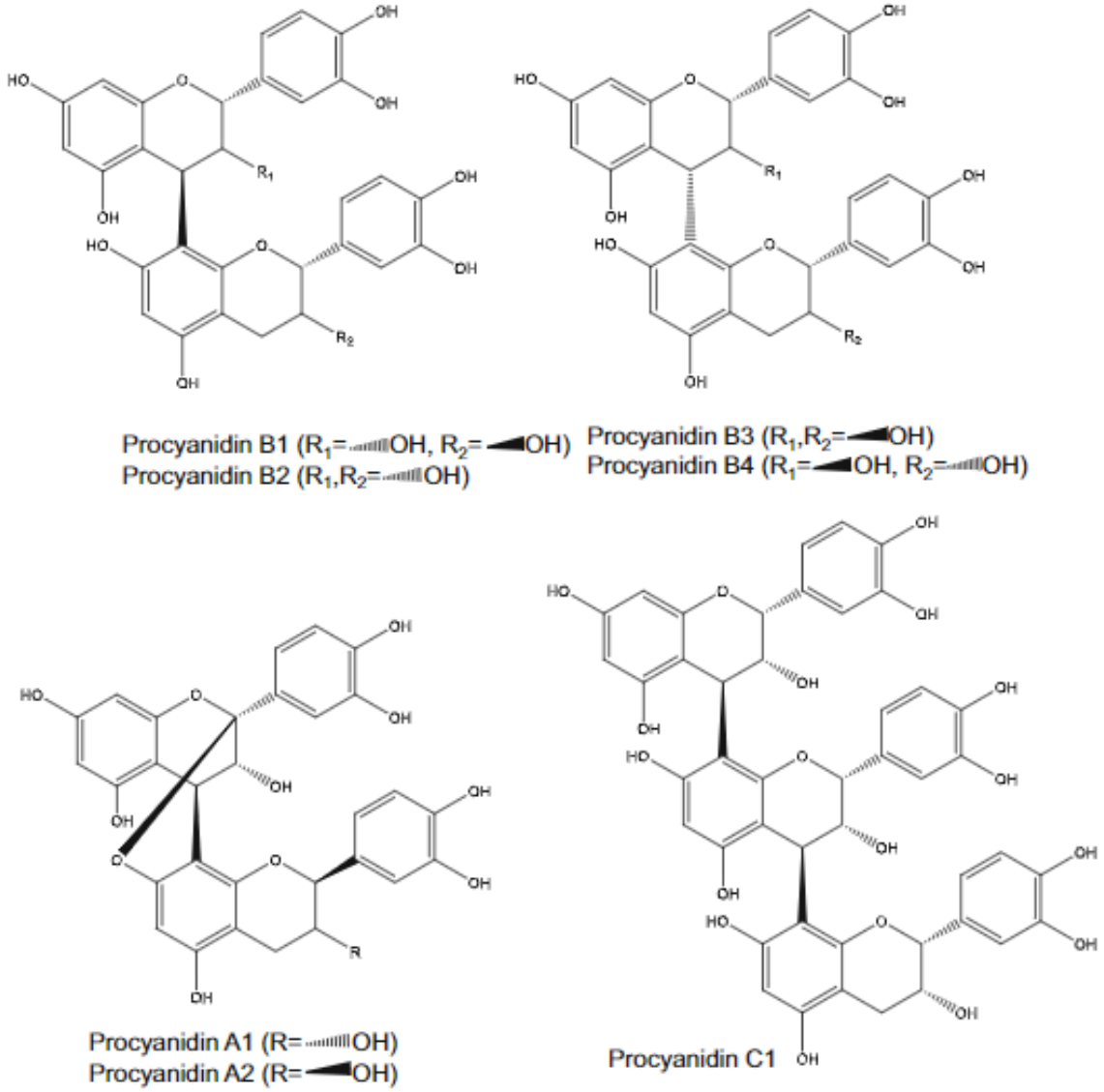

***Figure 3**: Chemical structure of monomeric and polymeric procyanidins* [6]

1.2.2. Flavones and flavonols

Flavones are one of the largest groups of flavonoids which are natural bioactive compounds found in various plants. They play an important role in plant signaling and defense. Flavones consist of a double bond between C-2 and C-3 and a connection of B ring on C-2[9].

Another subclass of flavonoids, which are alike flavones, are flavonols. Similarly, to flavones, they have an oxo group at the C-4 and a 2,3 double bond on ring C. The difference between flavones and flavonols is that flavonols have a hydroxyl group on C-3 position [10].

Three types of flavones and flavonol derivates were found in leaves of *Fallopia japonica* and those were quercetin, luteolin, and kaempferol derivates [5].

Quercetin (Que) (Figure 4) is a flavonol that is usually found in fruits, vegetables, and other plant sources. Quercetin and its derivates are natural plant compounds that show promising and diverse bioactive effects. Research has shown that they have anti-inflammatory, antioxidant, antimicrobial, antidiabetic, anticancer, and even cardiovascular and wound-healing properties. These compounds can be commonly found in Western diets such as apples, grapes, berries, cherries, citrus fruits, onions, kale, tomatoes, black tea, and red wine [11].

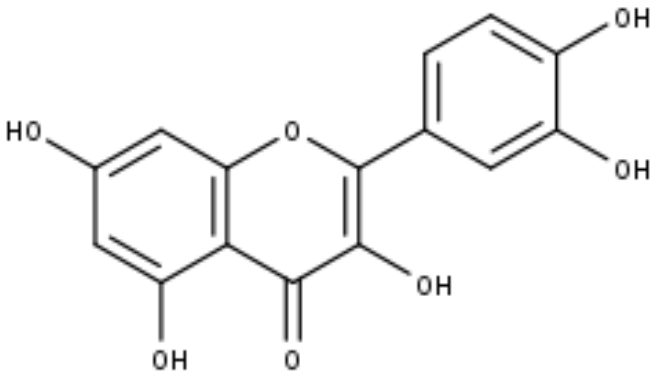

***Figure 4**: Chemical structure of quercetin*

Luteolin (Figure 5) is a flavon that is usually presented as a yellow crystal, and it can be found in many plants, fruits, and vegetables. As quercetin, luteolin and its derivates have many promising health benefits such as anti-inflammatory, antiviral, antioxidant, anti-cancer, heart protective, and anti-aging properties [12].

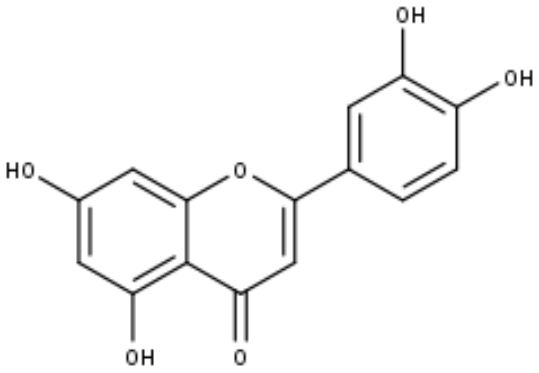

***Figure 5**: Chemical structure of luteolin*

Kaempferol (Figure 6), also known as kaempferol-3 or kaempferide, is a natural flavonol found in numerous vegetables and fruits such as beans, broccoli, grapes, strawberries, citrus fruits, and many others. It has proven to have many advantages to health such as anti-inflammatory and anti-cancer properties. Also, it plays an important role in improving liver injury, diabetes, and obesity. Kaempferol has the potential to inhibit vascular endothelial inflammation, maintain heart function, protect cranial nerves, and treat fibroproliferative conditions [13].

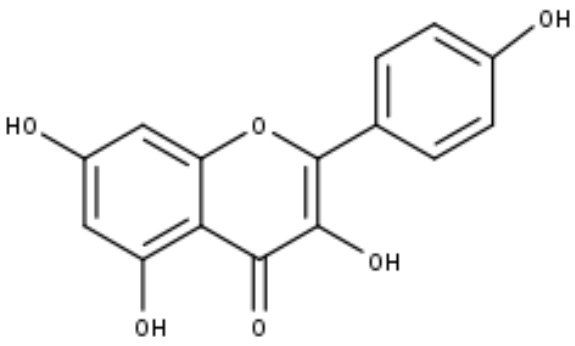

***Figure 6**: Chemical structure of kaempferol*

1.2.3. Phenolic acids

Phenolic acids are the main class of polyphenols which only have one carboxylic acid group. They are found in various kinds of foods like seeds, leaves of vegetables, and skins of fruits. Phenolic acids can be classified into two sub-classes: hydroxybenzoic and hydroxycinnamic acid. Hydroxybenzoic acids are derived from benzoic acids and are found in low concentrations in red fruits and vegetables like onions and black radishes. On the other hand, hydroxycinnamic acid is derived from cinnamic acid and is often found in foods as simple esters with glucose or quinic acid [14].

In *Fallopia japonica*, one of the major compounds found in the leaves is caftaric acid[5]. Caftaric acid (Figure 7) is a plant-derived compound classified as hydroxycinnamic acid. It is formed by the union of caffeic acid and tartaric acid. Also, it is present in high concentrations in all types of grape seeds and juices. Grape juice has shown anti-inflammatory and antioxidant effects due to the presence of caftaric acid in it. Also, caftaric acid can be useful in the treatment of hypertension and diabetes because it decreases high blood pressure and high blood glucose levels. Besides this, caftaric acid has been shown to have many other pharmacological effects that could be valuable for human health [15].

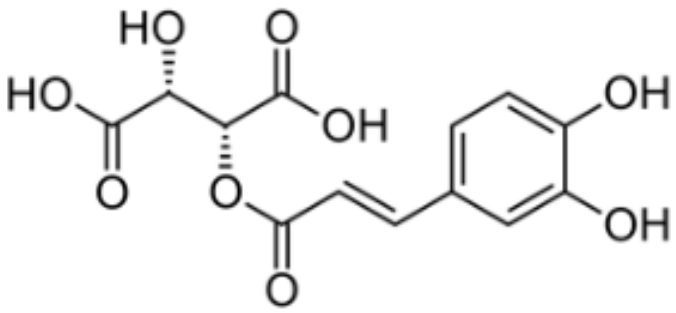

***Figure 7**: Chemical structure of caftaric acid* [15]

1.2.4. Triterpenoids

Triterpenoids are a group of natural compounds that have been studied for their potential therapeutical applications due to their diverse biological activities. Among many, ursolic acid and oleanolic acid seem to be the most present in leaves of *Fallopia japonica*[5].

Ursolic acid (Figure 8) is a pentacyclic terpenoid and secondary plant metabolite. It is usually present in stem bark, leaves, and fruit peel. It displays many pharmaceutical properties like the prevention of cancer that seem to capture a lot of attention. It suppresses transformation, induces apoptosis of tumor cells, and inhibits proliferation. As well as anti-cancer properties, ursolic acid has antioxidant, anti-inflammatory properties, and protective effects on internal organs against chemical damage. Although it has many health benefits, in clinical trials its application has reached limitations due to its poor bioavailability and absorption. However, the development of ursolic acid derivates seems to show improved antiproliferation activity. Therefore, its derivates demonstrate a possible solution in treating cancer that still needs more research [16,17].

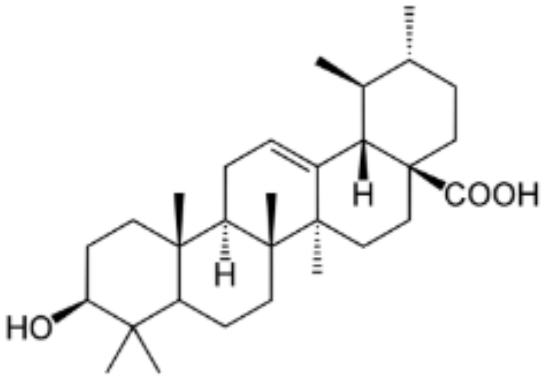

***Figure 8**: Chemical structure of ursolic acid* [16]

Oleanolic acid (Figure 9) is a pentacyclic triterpenoid that is widely distributed along the plant kingdom. It can be found in different foods and plants as free acid or as an aglycone of triterpenoid saponins. Oleanolic acid is non-toxic, and it has hepatoprotective, antitumor, antidiabetic, antioxidant, and antiviral properties. Yet, oleanolic acid has been considered a poorly bioavailable biomolecule due to its very low water solubility. Recent research has shown that its bioavailability can be increased when administered dissolved in lipophilic matrices. Even though oleanolic acid exhibits anti-HIV and anti-HCV activities, those activities were too low *in vitro*. However, new studies are investigating more potent synthetic analogs. Nevertheless, oleanolic acid represents a drug molecule with the potential to act against different diseases [18,19].

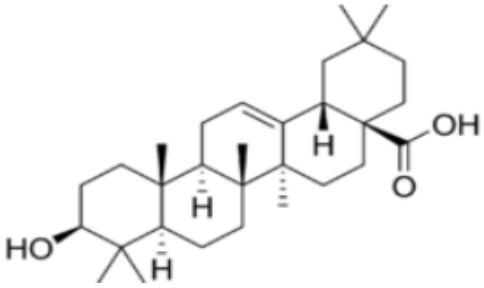

***Figure 9**: Chemical structure of oleanolic acid* [18]

1.3. Extraction of *Fallopia japonica*

Extraction, as an initial step, involves isolating the desired component from the raw material by using a solvent. Extraction is the crucial step in the preparation of the sample for further analysis of phenolic compounds and immensely depends on it. Phenolic compounds can exist in different kinds of analogous and they can vary through different parts of plants. Parameters that influence the success of antioxidant extraction are the type of solvent and its concentration, temperature, time of extraction, pH, characteristics of the phenolic compounds, preparation of the sample, and the ratio between the amount of the solvent and the material. Hence, it is important to select an appropriate extraction method to recover the desired phenolic compounds[12].

1.4. Measuring antioxidant activity

Oxidation processes are important for cells to survive. Organisms that undergo aerobic cellular respiration get energy from molecules like glucose. However, this process also creates free radicals which cause cellular damage. Free radicals contain an unpaired electron that makes them highly reactive. They are closely associated with oxidative stress. Oxidative stress occurs when there is an excess of reactive oxygen species (ROS) produced by the mitochondria in cells. Under normal conditions, the amount of ROS created and the amount that is removed are balanced. However, when there is a disbalance between pro-oxidants and antioxidants, it leads to oxidative stress.

Antioxidants are compounds that slow down and prevent or delay the oxidation of other molecules. During the process of oxidation, electrons or hydrogen atoms are transferred from the substance to the oxidizing agent. It can also occur the creation of free radicals which then kick off a chain reaction in the cell and cause damage to it. On the other hand, antioxidants prevent these chain reactions by removing the free radical intermediates and slowing down other oxidation processes. There is a growing interest in antioxidants from food scientists, health professionals, and the public due to their protective roles in food products against oxidative deterioration and their health benefits for pathogenic diseases caused by ROS.

In the last few decades, various assays have advanced in order to measure antioxidant activity such as single electron transfer (SET), hydrogen atom transfer (HAT), reducing power, metal chelation assay, and many others.

Radical/ROS scavenging involves a diverse group of assays in which antioxidants scavenge specific types of radical species. Depending on the chemical reactions, these assays can be divided into 2 groups: hydrogen atom transfer (HAT) reaction-based assay and single electron transfer (SET) reaction-based assay. The HAT-based assay measures the ability of

an antioxidant to bind to free radicals by donating a hydrogen atom. The reaction that occurs between phenolic antioxidant and peroxyl radical is (1):

$$ROO\bullet + AH/ArOH \rightarrow ROOH + A\bullet/ArO\bullet \qquad (1)$$

Aryloxy radical (ArO•) which is formed from this reaction is stabilized by resonance. To prevent oxidation, phenolic antioxidants had to react faster than biomolecules with the free radicals. In fluorescence methods, antioxidant and fluorescent probes react with a free radical. Antioxidant activity can be evaluated from competing rates between antioxidant and fluorescence probes since they both react with peroxide radicals. This involves comparing the fluorescence decay curve of the probe when the antioxidants are absent and present, integrating the area under these curves, and calculating the difference between them. However, single electron transfer (SET) based assay measures the ability of antioxidants to bind to free radicals by donating an electron. In the SET mechanism, an interaction takes place between the antioxidant and the colored probe (oxidized agent) or fluorescent rather than with peroxide radical. Spectrometric SET methods are based antioxidant's ability to reduce an oxidant and then cause the change in initial color. The degree of color change correlates with the concentration of antioxidants present in sample [20].

1.4.1. DPPH assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay is one of the numerous analytical methods that measure the antioxidant capacity mostly in plant and food extracts. It is based on spectrophotometric measurement of antioxidants` ability to scavenge DPPH radicals. The DPPH• is reduced to hydrazine by taking hydrogen atom from the antioxidants. When the DPPH• is mixed with an antioxidant, the dark purple color changes to pale yellow and results in a reduced form of DPPH radical (DPPH-H). The

formation of hydrazine (DPPH-H) is a result of radical reduction by hydrogen atom from antioxidant (Figure 10).

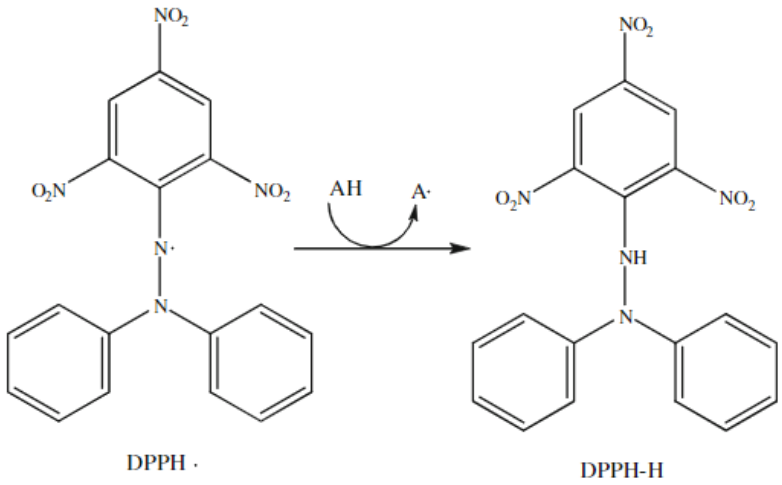

***Figure 10**: Antioxidant (AH) scavenging DPPH radical* [21]

The DPPH assay is mainly based on hydrogen atom transfer (HAT) and single electron transfer reaction (SET). However, this radical can be used to explore how phenols scavenge free radicals. It is now widely recognized that the interaction between phenols and DPPH occurs in two different mechanisms: direct hydrogen atom transfer (HAT) and sequential proton loss electron transfer (SPLET) mechanism. The 7-OH group in flavonoids has a crucial part as the site of electron transfer and of ionization in the sequential proton loss electron transfer (SPLET) mechanism. This mechanism has been discovered recently and it consists of two steps. First is deprotonation of antioxidant and second is an electron transfer from the deprotonated antioxidant to the free radical. The mechanism of HAT and SPLET between phenol and DPPH is following:

$$Ar\text{-}OH + DPPH\bullet \rightarrow Ar\text{-}O\bullet + DPPH\text{-}H \qquad (HAT)$$

$$Ar\text{-}OH \rightarrow Ar\text{-}O^{-} + H^{+} \qquad (SPLET)$$

$$Ar\text{-}O^{-} + DPPH\bullet \rightarrow Ar\text{-}O\bullet + DPPH^{-}$$

$$DPPH^{-} + H^{+} \rightarrow DPPH\text{-}H$$

DPPH radical is a stable radical due to the delocalization of the spare electron in the whole molecule (Figure 11). This delocalization causes a dark purple color to appear in the molecule. The reducing ability of antioxidants towards DPPH is determined by measuring the absorbance at 517 nm by UV-Vis spectrophotometer. In this assay, the absorbance is measured before and after the antioxidant has been added. The reduction of absorbance is a measure of the free DPPH• as a result of the action of the antioxidant [21,22].

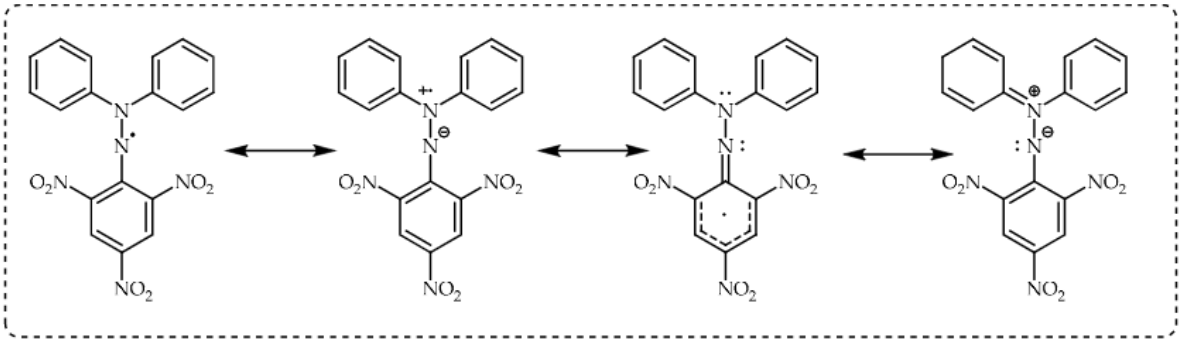

***Figure 11**: The resonance structures of 1,1-diphenyl-2-picrylhydrazil radical (DPPH•)* [22]

The DPPH assay is an easy, simple, rapid, and economical assay for measuring the antioxidant activity of phenolic compounds. Several factors can influence its sensitivity, including type and amount of used solvent, freshness of DPPH reagent, presence and concentration of hydrogen and metal ions. However, the DPPH assay has one large limitation. Certain compounds absorb light within the same wavelength range as DPPH radical. For instance, anthocyanins have intense absorption in a similar wavelength range (500-550 nm) as DPPH radical. This can potentially interfere with the results and their interpretation [23].

1.4.2. Folin-Ciocalteu Method

The Folin-Ciocalteu test is for determining the total phenolic content (TPC). It is widely used for measuring total polyphenolic content in plants and food materials. It is important to note that total phenolic content is not an antioxidant assay. However, the high quantity of phenolic content is correlated to high antioxidant ability. Therefore, the total phenolic

content is important for the determination of total antioxidant ability. This method was initially designed to analyze proteins but later it was modified to analyze the phenolic components in wine and subsequently became a routine test for antioxidant estimate of plant extracts and food [23].

The Folin-Ciocalteu test is based on the reduction of the Folin-Ciocalteu reagent with phenolic compounds under alkaline conditions. The actual mechanism of the Folin-Ciocalteu reagent remains unclear. However, it is hypothesized that it contains phosphomolybdic/phosphotungstic acid which undergoes a reaction to achieve a blue chromophore with the highest absorption at 765 nm (Figure 12). The central molybdenum ion with this complex is recognized as the reducing site, wherein the Mo6+ ion undergoes reduction to Mo5+. This reduction is obtained by accepting an electron from the phenolic antioxidant and that causes the color development. Hence, the Folin-Ciocalteu test relies on the SET mechanism in an alkaline medium from phenolic compounds and other reducing compounds to molybdenum, consequently forming blue complexes that can be detected spectrophotometrically at 765 nm.

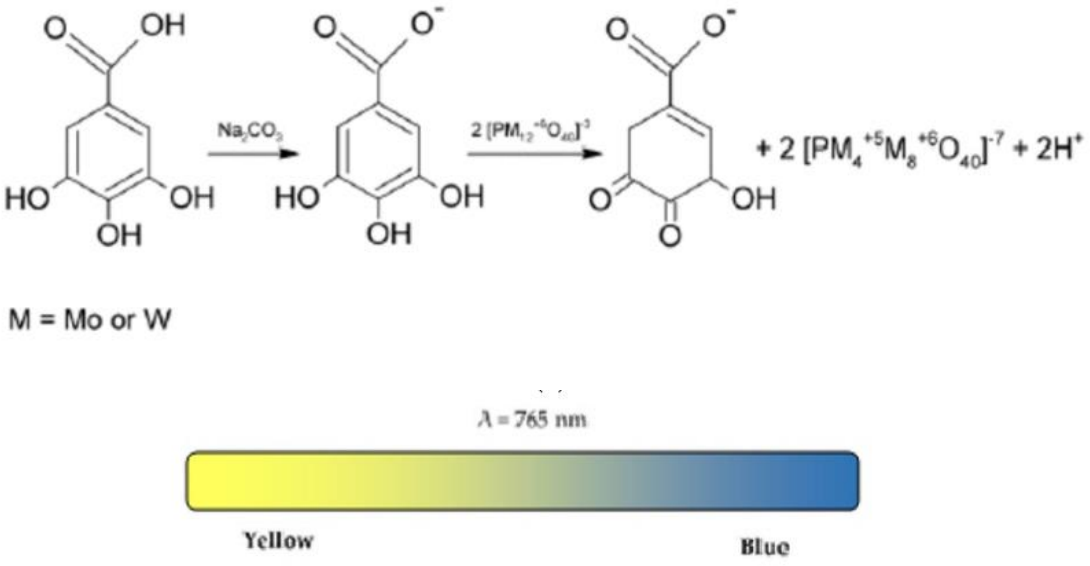

***Figure 12**: The chemical reaction between phenolic antioxidant and Folin-Ciocalteu reagent in alkaline solution, resulting in the formation of blue color* [24]

The Folin-Ciocalteu test for measuring TPC has many advantages such as simpleness, robustness, and reproducibility. Nonetheless, it also has some disadvantages. The downside of the Folin-Ciocalteu test is that the test is sensitive to temperature, pH, and reaction time. Also, the results from this test can be overestimated by one size as to those obtained by HPLC methods [24].

1.4.3. Cellular antioxidant assay (CAA)

The cellular antioxidant assay is a cell-based assay for measuring antioxidant activity within a cell in a standard cell culture environment which includes temperature, uptake, pH, metabolism, and antioxidant capability. This assay uses a fluorescent probe (2',7'-dichlorofluorescein diacetate (DCFH-DA)) to oversee the peroxyl radical triggered oxidation within the cell. The ester form of the dye (DCFH-DA) is selected due to its nonionic and nonpolar nature which allows transport through the cell membrane. Once inside the cell, endogenous cellular esterase deacetylates DCGH-DA and converts it into a more oxidizable DCFH form (Figure 13).

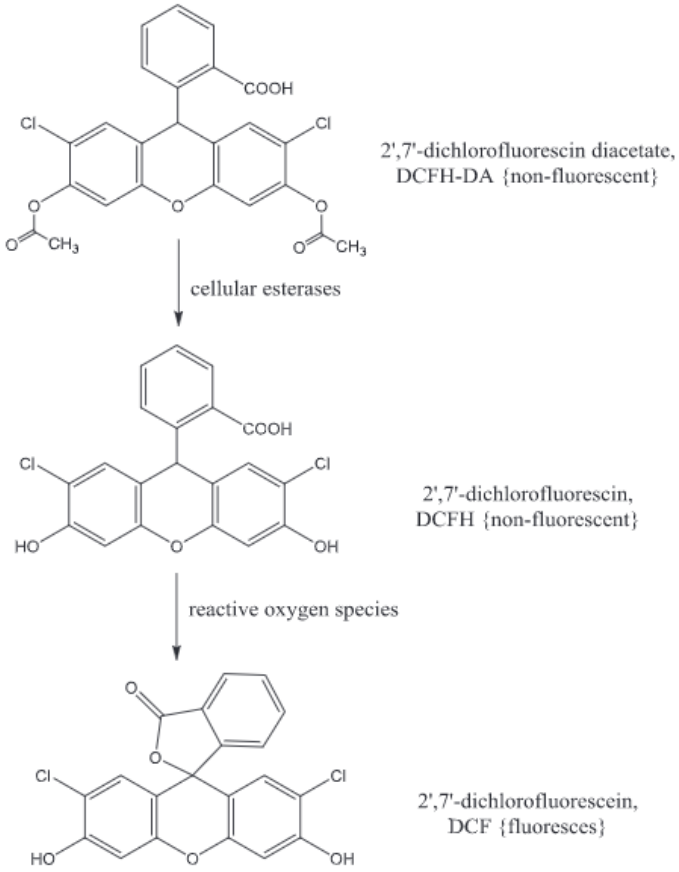

***Figure 13**: Chemistry of 2',7'-dichlorofluorescein diacetate (DCFH-DA) as it enters the cell* [25]

ABAP or 2,2'-azobis(2-amidinopropane) dihydrochloride, a free radical initiator, is added and it induces peroxyl radicals. Other reactive oxygen species (ROS) are produced through cellular processes. When antioxidants penetrate the cell, they compete with radicals and quench them through various mechanisms (Figure 14). This prevents DCFH from oxidizing and forming the 2',7'-dichlorofluorescin form (DCF). The whole impact of these pathways is evaluated by observing the intracellular fluorescence from DCF over a timeframe: reduced fluorescence relative to a control (without antioxidant compounds) indicates considerable cellular antioxidant activity. In its early stages, the assay relied on PC12 cells obtained from rat adrenal glands. Nowadays, it is common to find references in scientific literature to use HepG2 cells, which are derived from human hepatocellular carcinoma [25].

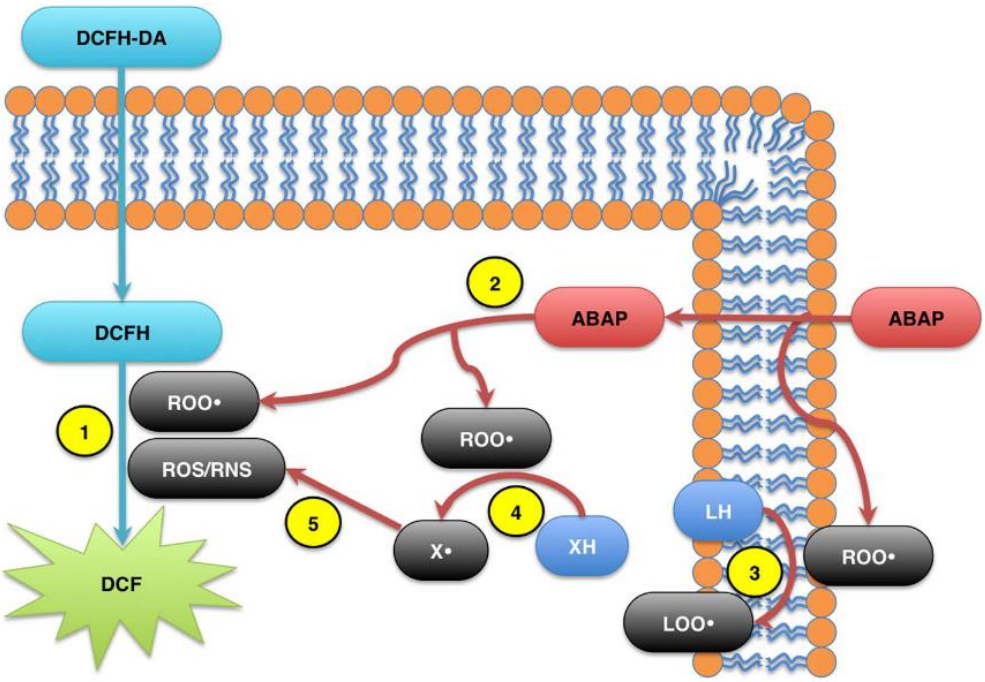

***Figure 14**: The potential ways in which antioxidants can function when they are incubated with 2',7'-dichlorofluorescein diacetate (DCGH-DA) include: (1) quenching free radicals before they can reach 2',7'-dichlorofluorescein (DCFH) dye; (2) directly reacting with 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) to inhibit the formation of radicals; (3) inhibiting lipid peroxidation; (4) Reacting with alkyl peroxyl radicals to interfere with the propagation of other radicals; (5) Blocking intracellular redox pathways that could also oxidize the DCFH* [25]

1.5. Lactic acid fermentation

Fermentation is a process of transforming large organic molecules, such as sugars, into molecules with additional biological value through the action of microorganisms. One of the dominants used in food production is lactic acid fermentation performed by lactic acid bacteria (L.A.B.). They produce lactic acid during the fermentation of carbohydrates and play a critical role in the production of desirable biochemical changes in fermented foods/beverages. They include genera such as *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* which are used as a starter culture in various fermented foods [26]. Lactic acid fermentation causes modifications in foods' composition due to the several mechanisms caused by molecules such as bioactive peptides, polysaccharides, and short-chain

fatty acids. During the fermentation, the sugar content and antinutritional compounds decrease, while phenolic compounds are modified into molecules with a nutritional advantage. Therefore, fermented foods, which consist of improved food components bioavailability, can be an additional or alternative source of food in an individual's daily consumption [27].

2. AIMS OF THIS THESIS

The main aim of this thesis is to determine the impact of extracts derived from both non-fermented and fermented Japanese knotweed (*F. japonica*) on oxidation level within the yeast *Saccharomyces cerevisiae*. The main hypothesis is that if lactic acid fermentation has an influence on the antioxidative capacity of Japanese knotweed, then there will be differences in chemical composition between non-fermented and fermented Japanese knotweed that would influence the antioxidant activity in the cells.

In order to assess the antioxidant properties of Japanese knotweed, different kind of methods were used: total phenolic content (TPC), DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging assay, and cellular antioxidant activity (CAA). The yeast *Saccharomyces cerevisiae* was used as a model organism in CAA assay due to its similarity of basic cellular processes and structure to more complex eukaryotes, including humans. These studies could provide a potential discovery regarding lactic acid fermentation influencing the chemical composition of Japanese knotweed and its potential to contribute more beneficial chemical profile in Japanese knotweed.

Hence, the sub-aims of this thesis are:

1. To follow the lactic acid fermentation of Japanese knotweed by measuring *Lactobacillus plantarum* growth and pH value,
2. To determine the total phenolic content (TPC) and *in vitro* antioxidant activity (DPPH scavenging assay) in extracts from non-fermented and fermented Japanese knotweed and compare them,
3. To determine the cellular antioxidant activity of extracts from non-fermented and fermented Japanese knotweed and compare them.

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

- MRS agar (Merck, Darmstandt, Germany)
- Agar (Biolife, Romania)
- Cycloheximide (Sigma-Aldrich, Germany)
- Ethanol (Sigma-Aldrich, Germany)
- Sodium chloride (Sigma-Aldrich, Germany)
- DPPH (Sigma-Aldrich, Germany)
- Methanol (Merck, Darmstandt, Germany)
- Folin-Ciocalteu reagent (Sigma-Aldrich, Germany)
- Sodium carbonate (Merck, Darmstandt, Germany)
- Trolox (Sigma-Aldrich, Germany)
- Chlorogenic acid (Sigma-Aldrich, Germany)
- Glucose (Merck, Darmstandt, Germany)
- Yeast extract (Merck, Darmstandt, Germany)
- Peptone (Merck, Darmstandt, Germany)
- Phosphate-buffered saline, PBS (Oxoid, UK)
- Dipotassium hydrogen phosphate (Kemika, Croatia)
- Monopotassium phosphate (Kemika, Croatia)
- Dimethyl sulfoxide, DMSO (Sigma-Aldrich, Germany)
- 2',7'-dichlorofluorescein diacetate, DCFH-DA (Sigma-Aldrich, Germany)

3.2. Instruments

- Analytical balance ME204 (Mettler toledo)
- Scale PFB 2000-2 (Kern)
- Steam sterilizer (Sutjeska)
- Incubator (Kambich)
- Magnetic stirrer (Tehtnica)
- Multitron shaker (Infos HT)
- Vortex TTS2 (Yellow line)

- Vibromix 10 mixer (Balance)
- pH meter SevenMulti (Mettler toledo)
- Dust-free chamber LFVP 15 (Iskra Pio)
- Centrifuge Centric200 (Tehtnica)
- Centrifuge Centric322A (Tehtnica)
- UV-VIS Spectrophotometer 89090A (Agilent)
- Rotavapor vacuum concentrator 2-33 CDplus (Martin Christ) + Vacuum system MD 4C (VacuumBrand)
- Water bath with shaking SW-21C (Julabo)
- Ultrasonic bath
- Chamber IG150 (Jouan)
- Safire2 reader (Tecan)

3.3. *Lactobacillus plantarum* inoculum preparation

Lactobacillus plantarum (IM 527) was obtained from the Institute of Dairy Science and Probiotics, Biotechnical Faculty, Ljubljana.

An initial culture preserved in 20% (v/v) glycerol was transferred into 20 ml of sterilized MRS broth. Next, an overnight incubation was carried out using a rotary shaker (operating at 30°C and 150 rpm). After overnight incubation, 2 mL of culture was centrifuged at 10000 rpm for 5 minutes. The supernatant was removed, and 2 mL of sterile physiological solution (0.9% (w/v) NaCl) was added. After mixing it on a vortex, the sample was centrifuged again under the same conditions. Once again, the supernatant was removed, 2 mL of sterile physiological solution was added, and mixed on the vortex.

3.4. Preparation of Japanese knotweed (*Fallopia japonica*)

Dried and pulverized *Fallopia japonica* was obtained from the Department of Agronomy, Biotechnical Faculty, Ljubljana.

3.5. Lactic acid fermentation

To create a non-fermented broth, an amount of 1 g of fresh lyophilized and pulverized *F. japonica* was mixed with 7 mL of sterile physiological solution immediately after the preparation. To non-fermented broth samples, a 1% (*v/v*) inoculum of *L. plantarum* was applied, initiating fermentation at 30°C for 72 h. Broth samples were collected at four time points: immediately after inoculation (t=0) and 24, 48, and 72 hours later. These samples were frozen for further analyses, except for the determination of *L. plantarum* growth which was conducted right away after each sample was taken.

3.6. *L. plantarum* growth determination

Both samples, fermented and non-fermented, were homogenized on a shaker and diluted according to the Koch method. These dilutions were further placed dropwise (10 µL) to MRS plates containing cycloheximide. The inoculated MRSc plates were placed in an anaerobic jar and incubated at 30°C for 48 h. After the incubation period, the number of colonies was counted, and the results were expressed as the logarithm of the number of colony-forming units (CFU) per mL of broth, denoted as log CFU/mL.

3.7. Determination of pH

An amount of 1 g of broth was mixed with 9 mL of sterile distilled water. The suspension was homogenized, and the pH value was measured from the supernatant.

3.8. Preparation of extracts from non-fermented and fermented *F. japonica* for antioxidant activity determination

In order to determine the antioxidant activity and total phenolic content, extracts from fermented and non-fermented *F. japonica* were prepared using 70% ethanol.

A two-stage extraction was performed for both fermented and non-fermented samples. Since every sample contained a different amount of broth, the amount of extraction solvent was calculated for each sample individually. For the extraction of 500 µg of sample, it requires 750 µL of 70% ethanol for each step of extraction. For the first stage, the broth sample was weighed into 50 mL centrifuge tubes and mixed with the appropriate amount of 70% ethanol. The extraction process took place for 30 minutes in a water bath set at 40°C with shaking. Following this, the samples were centrifuged for 30 minutes at 6000 rpm, and the resulting supernatant was collected.

For the second stage, the remaining residues were extracted with another 12 mL of 70% ethanol, following the identical procedure as before. Both supernatants were combined to create a joint extract. To enhance the extraction yield, another centrifugation of the joint extracts was done for 10 minutes at 6000 rpm. This final extract was stored in a freezer at -20°C until further analysis, specifically to determine the total phenolic content and antioxidant activity.

To determine antioxidant activity in the cells, concentrated extracts were used. The ethanol extracts are subjected to evaporation after which they are freeze-dried.

The ethanol extracts were dissolved in DMSO using an ultrasonic bath to achieve concentrated extract with a dry extract concentration of 80 mg/mL.

3.9. Total phenolic content

The total phenolic content of *Fallopia japonica* extracts was determined using the Folin-Ciocalteu reagent, followed by spectrophotometric quantification. The calibration curve was prepared with 0 to 200 µL of a standard solution of chlorogenic acid (0.46 mM in 70% ethanol) and the corresponding amount of the solvent (70% ethanol) to obtain a total of 725 µL. Afterward, 125 µL of freshly diluted Folin-Ciocalteu reagent (diluted at a 1:2 ratio in water) was introduced and followed by the addition of 125 µL of 20% Na_2CO_3 (diluted in water) after precisely 5 minutes. After thorough mixing, the samples were incubated in the dark at room temperature for 60 minutes to complete the reaction. After incubation, the samples were centrifuged on a rotary centrifuge at 13000 rpm for 5 minutes, followed by measuring the absorbance against a blank and the samples at 765 nm. To evaluate the total phenolic content in extracts, 50 µL of the diluted sample (diluted at a 1:10 ratio in 70% ethanol) and 675 µL of water were analyzed using the same methodology. The results are expressed as equivalents of chlorogenic acid (mg) per mL of ethanol extract.

3.10. Antioxidant activity *in vitro*

Antioxidant activity *in vitro* was determined using the DPPH radical scavenging assay. The calibration curves were prepared from 0 to 50 µL of Trolox standard solution (1.14 mM in absolute ethanol) with a corresponding volume of 70% ethanol in order to reach a total volume of 50 µL. This mixture was then mixed with freshly prepared 0.11 mM DPPH• in methanol. The samples were incubated in the dark at room temperature for 1 hour. After incubation, the samples were centrifuged on a rotary centrifuge at 13000 rpm for 5 minutes. The absorbance of the reaction mixture was measured at 517 nm. To evaluate the antioxidant activity in extracts, 10 µL of diluted sample (diluted at a 1:10 ratio in 70% ethanol) and 40 µL of 70% ethanol were used to analyze following

the same protocol. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) μg per liter of ethanol extract.

3.11.Cellular antioxidant activity (CAA) Assay

Cellular antioxidant activity was determined by measuring intracellular oxidation in *Saccharomyces cerevisiae*, serving as a model organism. The yeast *Saccharomyces cerevisiae* was cultivated in YEPD medium at 28°C for 3 days. Subsequently, the cells were centrifuged on the rotary centrifuge for 4 min at 3000 rpm, the supernatant was removed, and 50 mL of PBS buffer was added. This process was repeated once more. From this mixture, 20 mL of culture was suspended in PBS buffer. The culture in PBS buffer was incubated for 24 h at 28°C and 220 rpm until the stationary phase. After incubation, 300 μL of concentrated ethanol extracts (80 mg dry extract/mL) of fermented and non-fermented Japanese knotweed were mixed with 9.7 mL of treated yeast cells and incubated for 2 hours in the same conditions. As controls, yeast cells were treated with the same volume of the DMSO. After treatment, the 2 mL of the cell suspension underwent centrifugation (14000 rpm, 5 min) and the supernatant was removed. Next, the cells were washed with 2 mL of 50 mM potassium phosphate buffer (pH 7.8), mixed, and centrifuged twice. Firstly, it was centrifuged for 2 min at 800 rpm from where the supernatant was centrifuged for 5 minutes at 14000 rpm. The supernatant was once again removed, and cell pellets were suspended in 250 μL of 50 mM potassium phosphate buffer. From this suspension, 100 μL was mixed with 890 μL of 50 mM potassium phosphate buffer. After a 10 min incubation at 28°C, 10 μL H_2DCFDA was added and incubated for 30 min at 28°C and 220 rpm. Following this, the fluorescence of the yeast suspension was measured using a Safire II microplate reader. The excitation wavelength for DCF was set at 488 nm, while the emission wavelength was 520 nm. Furthermore, the optical density of the yeast suspension was measured at 650 nm. The results were expressed as

relative values of fluorescence/optical density (F/OD) compared to the control (yeast cells treated only with DMSO).

3.12.Statistical analysis

Fermentation of *F. japonica* and every analysis were performed with a minimum of three repetitions. Differences between extracts from non-fermented and fermented broth samples for antioxidant assays (total phenolic content, antioxidant activity *in vitro,* and cellular antioxidant activity) were detected using the Student t-test. For the cellular antioxidant activity, a Student t-test was also used to detect differences between control and yeast cells treated with ethanol extracts of both fermented and non-fermented *F. japonica*. The differences were considered statistically significant when p-values were less than 0.05. Results are presented as means ± SD (standard deviation).

4. RESULTS

4.1. Lactic acid fermentation

Lactic acid fermentation of *F. japonica* inoculated with *L. plantarum* was followed by measuring the LAB growth through time which is shown in Figure 15. From Figure 15 we can see the rapid increase in the first 24 hours, its peak at 48 hours, and the lack of culture growth at 72 hours. At the inoculation (t=0), the culturability was 6.82 log CFU/mL and increased to 8.01 log CFU/mL after 48 hours.

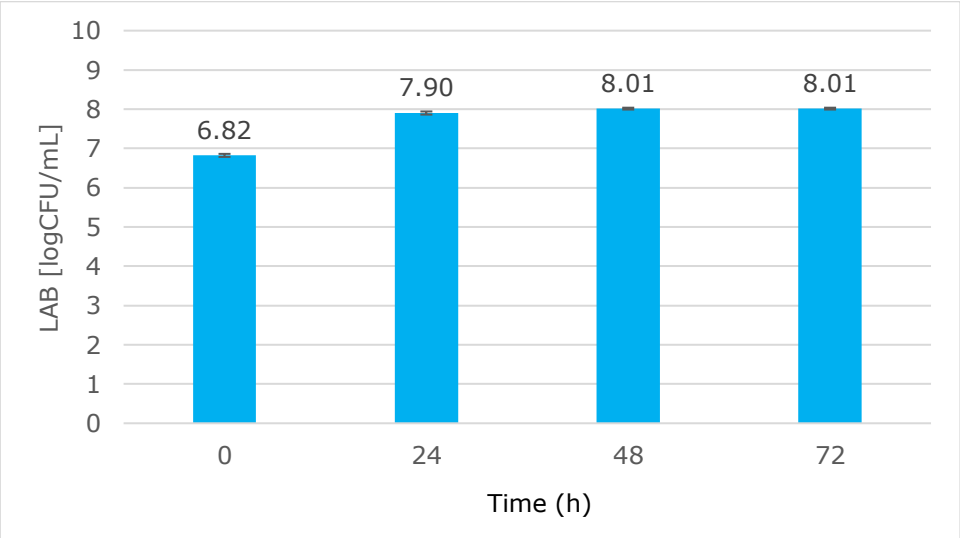

***Figure 15**: Determination of LAB concentration (LAB) before (0 h) and after fermentation (24, 48, 72 h). Data represent mean values ± SD (n =3).*

4.2. Determination of pH value

To determine the success of fermentation, the pH values were measured. The changes in pH values of lactic acid fermentation of *F. japonica* are shown in Figure 16.

The greatest change is observed in the first 24 hours when it decreases from 4.78 to 3.91. Considering standard deviation, the experimental results exhibit a consistent trend over the observed time period of 72 h.

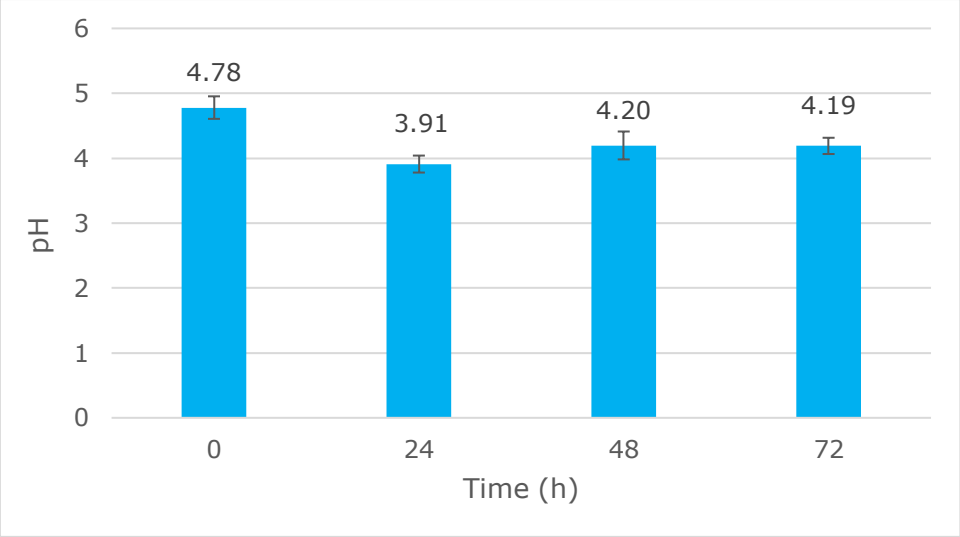

***Figure 16**: Evaluating the pH values of fermented (24, 48, and 72 h) and non-fermented (0 h) F. japonica. Data represents mean values ± SD (n=3).*

4.3. Total phenolic content

The total phenolic content (TPC) of ethanol extracts is shown in Figure 17. In the first 24 hours, there is no change in TPC levels. However, after 48 hours a significant decrease of 23% was observed, compared to extracts from non-fermented *F. japonica* (from 281.45 mg CHA/mL before fermentation to 217.32 mg CHA/mL (Table 1)).

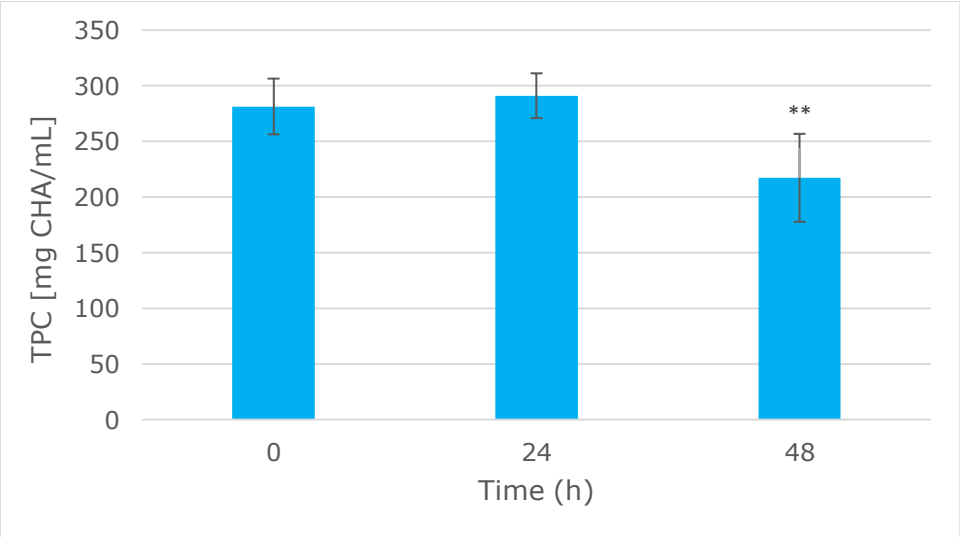

***Figure 17**: Total phenolic content of ethanol extracts from fermented (24 and 48 h) and non-fermented (0 h) F. japonica. Data demonstrate mean values ± SD and asterisks (**) represent statistically significant differences between ethanol extracts from fermented and non-fermented samples (p<0.1)(n=3).*

4.4. Antioxidant activity *in vitro*

Figure 18 presents ethanol extracts' ability to scavenge DPPH radicals. After 48 hours, an increase of 16% in TEAC of ethanol extracts from fermented *F. japonica* was observed (from 3.61 µg/L before fermentation to 4.32 µg/L (Table 1)) compared to the ethanol extracts from non-fermented *F. japonica* (0 h). After 24-hour fermentation, there is no increase in TEAC levels.

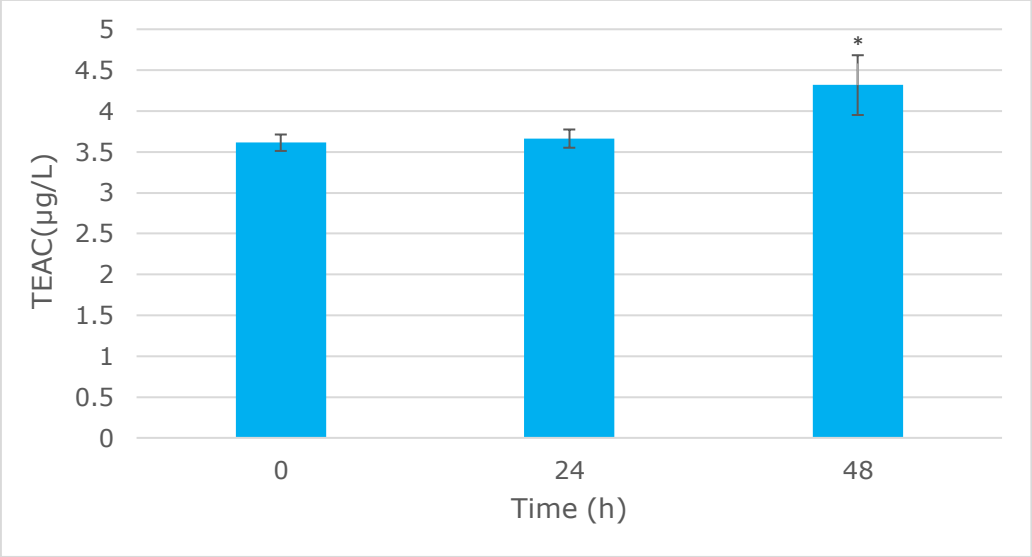

***Figure 18**: Total equivalent antioxidant activity (TEAC) of ethanol extracts of fermented (24 h and 48 h) and non-fermented (0 h) F. japonica. Data demonstrate mean values ± SD and asterisk (*) represent statistically significant differences between ethanol extracts from non-fermented and fermented Japanese knotweed ($p<0.05$)($n=3$).*

4.5. Cellular antioxidant activity

Yeast *Saccharomyces cerevisiae* was used as a model organism for the determination of cellular antioxidant activity of ethanol extracts from fermented and non-fermented *F. japonica.* Results are shown in Figure 19 and Table 1. The most significant decrease in cell oxidation level compared to the control is observed after 48 h where the value reduces to 64.7%. At 0 h, the cell oxidation level decreases to 74.3%, and at 24 h, it reduces to 69.9%. If we compare the antioxidant activity of extracts from fermented and non-fermented samples, there is no significant difference.

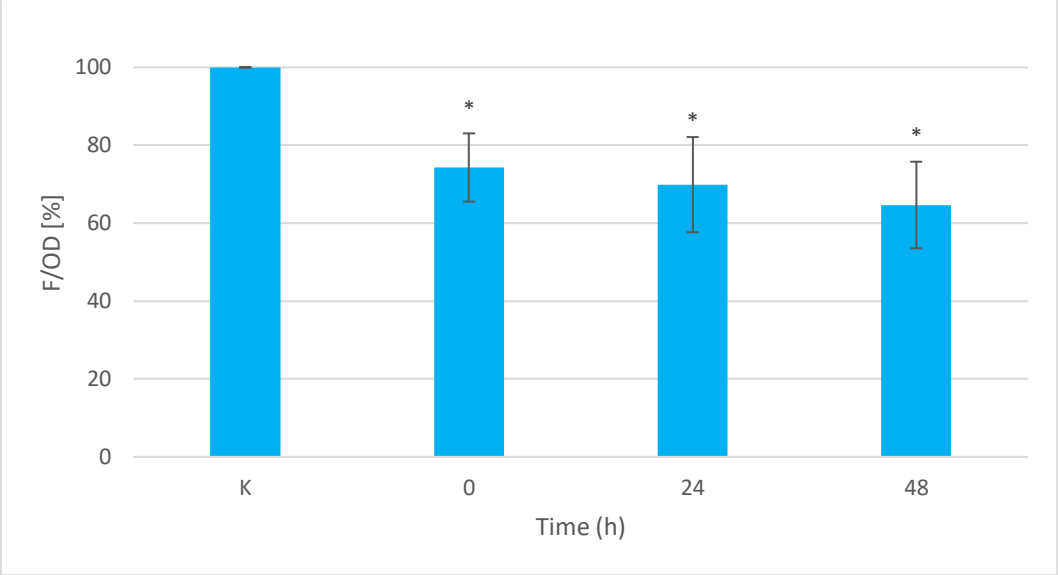

***Figure 19**: Measurement of intracellular oxidation of ethanol extracts of non-fermented (0 h) and fermented (24 and 48 h) Japanese knotweed in yeast Saccharomyces cerevisiae. Data demonstrate mean relative values F/OD to the correlating control set as 100%. Asterisk (*) represents statistically significant differences between control and yeast cells treated with extracts from non-fermented and fermented F. japonica (p<0.05)(n=3).*

***Table 1**: Representation of values of total phenolic content (TPC), total equivalent antioxidant activity (TEAC), and intracellular oxidation (F/OD) of ethanol extracts of non-fermented and fermented F. japonica.*

	Time (h)		
	0	**24**	**48**
TPC [mg CHA/mL]	281.45 ± 25.05	291.11 ± 20.15	217.32 ± 39.53**
TEAC(µg/L)	3.61 ± 0.1	3.66 ± 0.11	4.32 ± 0.37*
F/OD [%]	74.3 ± 8.8*	69.9 ± 12.2*	64.7 ± 11.1*

5. DISCUSSION

Before evaluating the differences in antioxidant activity of *Fallopia japonica*, it was necessary to ferment the Japanese knotweed. The aim of this work was to determine whether is there a difference between ethanol extracts from non-fermented and fermented *F. japonica* in terms of antioxidant activity.

Based on the results of lactic acid fermentation, the most significant changes in *L. plantarum* growth occurred during the first 48 h where we observed an increase from 6.82 log CFU/mL to 8.01 log CFU/mL. Also, the pH values show the most significant decrease in the first 24 h from 4.78 to 3.91. Similar results were seen with fermented *Arthrospira platensis* (*Spirulina*) where *L. plantarum* growth in the first 24 h reaches a maximal bacterial concentration of 8.5 log CFU/mL which stays the same through 48 and 72 h. Also, the pH decreased from 7.3 to 5.1 and remained constant in the next 48 h [28]. The process of fermentation and determination of pH and *L. plantarum* growth was done using the same method as for *F. japonica.* Therefore *F. japonica* was shown to be a successful substrate for fermentation and *L. plantarum* growth.

Other researchers reported similar results with the fermentation of liquorice root extract [29]. They investigated the effect of fortified liquorice root extract with different concentrations of high fructose corn syrup (HFCS) on physicochemical and functional properties. The pH analysis of all fortified samples conducted over 72-hour fermentation revealed a rapid decline in pH levels during the first 24 hours. In all samples of liquorice root extracts, there was a similar trend of minimal changes in pH levels in later stages by a gradual decrease of around 0.5 units in the next 48 hours. The decrease in pH is linked to the generation of acidic metabolites and a reduction in the medium's buffering capacity which occurs due to the rapid bacteria growth during the exponential phase. These findings support the results of fermented *F. japonica* where the correlation of increased LAB growth and lower pH value was demonstrated. Increased

LAB growth within the first 48 hours resulted in the production of acidic products such as lactic acid, thereby contributing to a decrease in pH levels. Likewise, the absence of culture growth consequently led to no change in pH levels. Given the absence of any observable alterations between 48 h and 72 h in *L. plantarum* growth and pH values, we chose not to incorporate the latter into our subsequent analysis.

After fermentation, the sample of *F. japonica* underwent a two-stage extraction with 70% ethanol. According to Naumoska et al.[30], the lyophilized and pulverized rhizome bark extract of *Fallopia japonica* were treated with different types of solvent mixtures. Those were distilled with water, methanol, 80% methanol$_{(aq)}$, 70% ethanol$_{(aq)}$, ethanol, acetone, 70% acetone$_{(aq)}$, and 90% ethyl acetate$_{(aq)}$. Results showed that the extraction yield was the highest with 70% ethanol$_{(aq)}$ and it even increased with the second extraction.

We further checked whether the fermentation was also reflected in the change in total phenolic content (TPC). Results from TPC demonstrate a significant decrease after 48 h of fermentation from 281.25 to 217.32 mg CHA/mL compared to extracts from non-fermented Japanese knotweed, which is a decrease of 22.8%. Li et al.[31] have similarly shown the reduction of total phenolic content of 22% in fermented apple juice treated with *L. plantarum*. As they explain, one of the reasons for the decrease in TPC can be due to the presence of lactic acid bacteria, which can depolymerize complex phenolic compounds into simple phenolic molecules and further convert them. In addition, this reduction in TPC did not contradict the observed increase in the antioxidant activity of the fermented apple juice. The reason behind this contradiction lies in the possibility that lactic acid bacteria consumed the glucose molecules within the phenolic compounds. These compounds were transformed into free aglycones that had a higher number of hydroxyl groups or lower steric hindrance to hydroxyl groups. Thus, these transformations led to the production of metabolites with increased antioxidant activity in the apple

juice. The results from this research align with the findings of Japanese knotweed, and the underlying cause for this similarity may be attributed to the same underlying reason. According to the previous research of Lachowicz et al.[5], the leaves from *F. japonica* seem to be a better source of polyphenolic compounds than its stalks or roots. The leaves show above-average content of catechin, epicatechin, quercetin, luteolin, and kaempferol. Also, it is known that the Folin-Ciocalteu reagent, which is used to assess TPC, has the ability to interact with substances other than polyphenols within the extracts. During fermentation, LAB may break down some of these compounds, contributing to the decrease in TPC values [28].

On the contrary, the results show an increase in the DPPH radical scavenging ability of extracts after 48 h fermentation of 16% compared to non-fermented Japanese knotweed. As previously stated in the study of Li et al.[31], the reason behind the increase in antioxidant activity in apple juice is possibly due to the transformations of simple phenolic compounds into new bioactive compounds during fermentation. Thus, these transformations led to the production of compounds with a greater potential for scavenging free radicals and, therefore, possess higher antioxidant activity. Also, these transformations during fermentations could lead to the production of less polar aglycones which have increased antioxidant activity in ethanol extracts. The proposed explanation may also apply to the results of the *in vitro* antioxidant activity of ethanol extracts from fermented and non-fermented Japanese knotweed. Furthermore, the results obtained from Japanese knotweed are in agreement with Nisa et al. [32], who showed that fermentation of rice bran with *L. plantarum* caused an increase in antioxidant activity after 48 h. This observed increase in antioxidant activity may be attributed to the action of hydrolytic enzymes, which work through the substrate and expose the free hydroxyl groups within the phenolic structure. Therefore, the substrate's antioxidant activity is increased due to the presence of

these free phenolic compounds. Besides mentioned catechin, epicatechin, quercetin, luteolin, and kaempferol, leaves of *F. Japonica* have been shown to be a higher source of caftaric, ursolic, and oleanolic acid than other morphological parts of the tested plants. These compounds exhibit strong antioxidant properties [5].

In vitro assays are commonly used to analyze the antioxidant capabilities of substances, but they fall short of accurately forecasting how these substances will function as antioxidants in living organisms. The underlying reason is that antioxidant defenses serve a broader purpose than merely neutralizing ROS. In order to protect the cells from oxidative stress, antioxidants have the capacity to activate antioxidant enzymes, influence gene expression, or alter cellular signaling pathways. Cell-based assays, such as cellular antioxidant activity (CAA) are gaining prominence as a more biologically relevant technique, bridging the gap between *in vitro* assays and animal feeding studies or human clinical trials [25]. We opted for the yeast *Saccharomyces cerevisiae* in the stationary phase as a model microorganism which is used for the study of oxidative stress in higher eukaryotic cells. Initially, we assessed the cell viability using the CFU method to examine whether the extracts had any negative effect on yeast growth. Since there were no changes in cell viability, we further evaluated the antioxidant activity within the cells. The results were consistent with the results of the DPPH antioxidant assay. After 24 and 48 h, there is a reduction in intracellular oxidation level compared to the control indicating antioxidative activity of extracts also in the cells. But if we compare ethanol extracts from non-fermented and ethanol extracts from fermented Japanese knotweed, there is no significant difference. Petelinc et al. [33], conducted a similar study involving yeast cells where they exposed the cells to propolis fractions with varying polarities. These fractions were obtained through solid-phase extraction of a crude propolis extract and were eluted using ethanol gradients ranging from 30%-70% (EL30-EL70). Their findings revealed that yeast cells treated with less

polar EL70 eluate exhibited the most substantial reduction in intracellular oxidation level which also showed the greatest *in vitro* antioxidant activity measured by DPPH free radical scavenging. Furthermore, cellular uptake of particular eluates was tested and the greatest cellular uptake of certain phenolic compounds was exhibited by eluate EL70 which could explain the highest observed antioxidant activity within the cells.

A limited number of scientific studies have been done on Japanese knotweed regarding its antioxidant activity. Pogačnik L. et al.[34], conducted an *in vitro* comparison of bioactivities of Japanese knotweed and Bohemian knotweed ethanol extracts (96% ethanol). The DPPH scavenging capacity assay results showed that rhizome extracts of *F. japonica* have higher antioxidant capacity than flower extracts. However, the results of the CAA did not align with the previously tested results from the DPPH assay. The CAA method determined in human hepatocellular adenocarcinoma derivated cells (HepG2), exhibited the highest values for rhizome extracts and the lowest for flower extracts. This observation could be attributed to the fact that the CAA method is considered to be a more biologically valid method compared to classical biochemical assays such as the DPPH assay.

Many different methods have been introduced to identify effective antioxidants and evaluate their antioxidant potential. However, these assays have undeniable advantages but also certain limitations. The DPPH radical scavenging assay is a widely used, simple, easy, cost-effective, and efficient assay for assessing antioxidant activity. Nonetheless, the DPPH radical scavenging assay is highly sensitive to environmental factors, lacks kinetics data, lacks specificity, and overestimates the antioxidant capacity of certain compounds. Although the TPC assay is considered to be precise, simple, sensitive, and effective in characterizing botanical samples, these disadvantages of the DPPH assay, can be also applied to the TPC assay for the phenolic content. Neither of these assays offers insight into which compounds show antioxidant activity [23].

Therefore, using High-Performance Liquid Chromatography (HPLC) would be a suitable approach for evaluating the total antioxidant activity of the individual compounds within the mixture.

Saccharomyces cerevisiae is widely employed as a model organism for analyzing oxidative stress and the protective mechanism of antioxidants due to its resemblance of fundamental cellular processes to complex eukaryotes. Compared to the chemical antioxidant assays, using yeast as a model system is more biologically relevant because it considers the cellular uptake and complex biochemical processes occurring in the cells. However, there are limitations to yeast models since dietary antioxidants behave differently in humans, particularly in terms of their effective concentrations, metabolism, and absorption [35]. Cigut et al. [36], analyzed the antioxidant properties and cellular uptake of propolis and its primary phenolic compounds in the yeast *Saccharomyces cerevisiae*. Even though the intracellular oxidation of 96% ethanol extracts of propolis decreased, only certain compounds demonstrated cellular uptake. Results of ethanol extracts of fermented *F. japonica* show decreased intracellular oxidation, however, it raises the question of which compounds can enter the cells. Further research on ethanol extracts of fermented Japanese knotweed should be carried out to gain deeper insight into how its antioxidant activity is exhibited in living organisms.

To the best of our knowledge, we did not find any existing studies that have compared *Fallopia japonica* biomass before and after fermentation with *L. plantarum*. Antioxidant levels of ethanol extracts of *F. japonica* seem to be influenced by fermentation and therefore, more attention should be paid to researching its antioxidant potential. Based on this analysis, further research could identify bioactive substances that exhibit these changes in antioxidant levels *in vitro* and *in vivo* of ethanol extracts of fermented *F. japonica*. Additionally, evaluating the nutritional quality and benefits of these compounds in ethanol extracts of fermented *F. japonica* could provide greater insight as a health-enhancing source.

6. CONCLUSION

The invasive nature of Japanese knotweed presents a challenge due to its negative ecological impacts. However, analysis of its chemical composition has revealed the presence of valuable active compounds that are known for their wide array of physical and chemical properties. The aim of this study was to provide the impact of extracts from non-fermented and fermented leaves of Japanese knotweed on oxidation level in the yeast *Saccharomyces cerevisiae*. The results show the fermentability of Japanese knotweed, confirmed by the proliferation of Lactic Acid Bacteria (LAB) and by reduction in pH levels. Compared to the extracts from non-fermented Japanese knotweed, the level of total phenolic content decreased in extracts from fermented Japanese knotweed. However, extracts from fermented *F. japonica* showed an increase in antioxidant levels *in vitro* and *in vivo*. The most significant reduction in cellular oxidation level compared to the control occurs after 48 h. Nonetheless, when we compare the antioxidant activity within cells of extracts from both fermented and non-fermented *F. japonica*, no significant difference is evident. Therefore, the antioxidant activity of ethanol extracts from fermented as well as non-fermented Japanese knotweed suggests the possible use of it in the pharmacological or food industry. Still, further research is needed to identify these bioactive compounds that show a decrease in oxidation level in order to support the promising prospects of this invasive plant as health-enhancing.

7. LITERATURE

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