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Studies of the Variability of Sugars, Vitamin C, and Chlorophylls in Differently Fermented Organic Leaves of Willowherb (*Chamerion angustifolium* (L.) Holub)

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Abstract: There is currently an increasing interest in functional foods and herbs as an opportunity to enrich one's diet and at the same time improve one's health. One of such plants is willowherb (*Chamerion angustifolium* (L.) Holub), which is rich not only in polyphenols, carotenoids, but also in sugars, chlorophylls, and vitamin C. This work purpose was to determine the effect of solid-phase fermentation (SPF) on changes in sugars, chlorophylls, and vitamin C under different fermentation conditions. Willowherb leaves were fermented for various durations (24, 48, and 72 h), in anaerobic and aerobic terms. The determination of sugars, chlorophylls, and vitamin C was done using high-performance liquid chromatography (HPLC) coupled to a spectrometer UV-VIS. The principal component analysis (PCA) was done to estimate the relationships between the different fermentation conditions (methods, as well as duration) and 10 parameters. The study showed: the biggest amounts of total chlorophylls and sugars were present after 72 h of anaerobic SPF, but the amount of total vitamin C was higher in unfermented willowherb leaves. In summary, SPF could be applied to modify chlorophylls and sugar quantities in willowherb organic leaves.

Keywords: fermented; chlorophyll; willowherb; vitamin C; glucose; fructose

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1. Introduction

People currently consume a lot of food, but often this food is nutritionally poor. As health is highly dependent on the quality of food consumed on a daily basis, it is especially important to choose foods, that contain more biologically active substances and a good ratio of sugars, vitamins and chlorophylls. Of course, it is also important whether the food is grown in a conventional way or organically. More and more people are choosing organically grown herbs, vegetables, and other products, because of their higher quality compared to conventional cultivation [1,2].

The willowherb (*Chamerion angustifolium* (L.) Holub) is widespread plant all over the world [3]. This plant is used both as food and as medicine. Due to its rich chemical composition, this useful plant can help to solve many nutritional and health problems [4,5]. It is very important to choose the right method of willowherb leaf preparation [6]. One of the methods of preparation of the *Chamerion angustifolium* leaves is solid-phase fermentation (SPF), which can be carried out under both aerobic and anaerobic conditions, and in various durations: 24, 48, or 72 h. Another significant element to set the final quantitative and qualitative composition of the raw material is the method of drying, which can be carried out in a conventional manner or by lyophilization [7].

Insufficient studies have been performed to determine how solid-phase fermentation under various conditions results in changes in the biochemical consistof willowherb leaves. This study purpose was to set the optimal conditions and duration of solid-phase fermentation to maintain the highest vitamin C, sugars, and chlorophylls contents in the leaves of the willowherb. Based on the obtained results, we could prepare recommendations as to how to use fermented products of willowherb, and provide recommendations to manufacturers to produce functional willowherb products.

2. Materials and Methods

2.1. Willowherb Leaves Samples

The willowherb organic leaves, that grew in the Jonava district, Safarkos village organic farm (55°00′22″ N 24°12′22″ E, Lithuania) of Giedre Naceviciene were collected and researched in 2020.

2.2. Willowherb Leaves Preparation

The willowherb organic leaves were collected at random from various places of experimental field at the beginning of mass flowering (July). The composite leaf sample was 6.3 kg. The leaf samples were divided into seven groups for the laboratory experiments:

1. Control—unfermented (NF): 0.900 kg.
2. Aerobic solid-phase fermentation (AEF): 2.7 kg for all the three SPF duration conditions lasting 24, 48, and 72 h.
3. Anaerobic solid-phase fermentation (ANEF): 2.7 kg for all the three SPF duration conditions lasting 24, 48, and 72 h.

Solid-phase fermentation (SPF) process: special plastic knives were used to cut fresh leaves. The samples were divided into 0.300 kg parts. For anaerobic solid-phase fermentation the leaves were tightly pressed into glass containers and covered with a cover. For aerobic solid-phase fermentation, containers (made from glass) were covered with an air-passing cover. The SPF took place in a dark chamber with a temperature of 30 °C for different length: 24, 48, and 72 h. These variants of the investigation were performed in three replications. Willowherb raw leaves were lyophilized in a freeze-drying plant sublimator (ZIRBUS GmbH, Harz, Germany), then these leaves were milled and stored in closed containers (temperature of 25 °C) in a dry, ventilated, cool and dark room. All chemical analyses were performed three times.

2.3. Vitamin C Analysis

Vitamin C was determined with an HPLC coupled to a spectrometer UV-VIS (Shimadzu Manufacturing Inc., Tampa, FL, USA). Freeze-dried plant powder in an amount of 100 mg was weighed in a plastic tube, and 5 mL of 5% metha-phosphoric acid was added. Then samples were shaken and extracted in an ultrasonic bath (10 min 30 °C, 5.5 kHz). Next, the samples were centrifuged (10 min, 6000 rpm, 0 °C). The supernatant was transferred into a clear HPLC-vial and 100 µL was used for analysis (injection) [8].

The following analysis parameters were used: mobile phase acetic buffer (pH 4.4) composed from two solutions: 0.1 M acetic acid (glacial, 99.9% purity) and 0.1 M sodium acetate. The mobile phase was prepared in volume proportions of 63:37 v/v. Isocratic flow was used with 1 mL min^{-1} [8].

For each analytical combination four replicates were made. Five injections of L-ascorbic acid and dehydroascorbic acid standards were prepared from the prepared standard solutions. The chromatogram was read, and individual compounds were identified based on the retention time of the standards.

2.4. Sugar Analysis

Ponder and Hallmann (2020) described the method for sugars evaluation [8]. An HPLC coupled to a spectrometer UV-VIS (Shimadzu Manufacturing Inc., Tampa, FL, USA) was used for this analysis. Powdered freeze-dried plant material in the amount of 100 mg was weighed in a plastic tube and 5 mL of 80% acetone was added.

The samples were mixed with a vortex and then extracted in an ultrasonic bath (10 min, 30 °C, 5.5 kHz). Next, the samples were centrifuged (10 min, 6000 rpm, 3 °C). The clear supernatant was separated and 1000 µL was transferred into an HPLC-vial.

The sugar compounds were separated under isocratic conditions with a flow rate of 1 mL min^{-1} using 80% acetone with deionized water. The total time of the analysis was 15 min.

The sugar compounds (glucose, fructose, sucrose) were identified by using 99.9% pure standards (Sigma-Aldrich, Poland) and the analysis times for the standards [8].

2.5. Chlorophylls Analysis

Chlorophylls (*a* and *b*) were measured by an HPLC (Polish agent Shimpol, Warsaw, Poland) method described by Ponder and Hallmann (2020) [8]. Sample preparation included extraction of 100 mg of freeze-dried sample with 100% acetone using an ultrasonic cold bath (10 min, 0 °C, 5.5 kHz). Then samples were centrifuged (10 min, 6000 rpm, 0 °C). One milliliter of supernatant was transferred into an HPLC vial.

The wavelength used for detection was 445–450 nm. The chlorophylls' concentrations were calculated using standard curves and the sample dilution coefficient and presented in mg per 100 g of dry matter.

2.6. Statistical and Multivariate Analysis

The ANOVA (a two-way analysis of variance) method, with software package STATISTICA, was used to perform statistical analysis of obtained data (Statistica 12; StatSoft, Inc., Tulsa, OK, USA). The results were presented as the mean with standard error. To estimate the statistical significance of differences between the means ($p < 0.05$) the Fisher's Least Significant Difference (LSD) test was used. The principal component analysis (PCA) was carried out to estimate the relationships between the different fermentation conditions (methods, as well as duration) and 10 parameters: vitamin C, dehydroascorbic acid, L-ascorbic acids, total chlorophyll, chlorophyll *a*, chlorophyll *b*, total sugars, fructose, glucose, and sucrose.

3. Results

3.1. The Amounts of Biologically Active Compounds

Fermentation duration and conditions (aerobic or anaerobic) produced quantitative changes in vitamin C, chlorophylls, and sugars. The highest content of vitamin C was in unfermented willowherb leaves (control), 678.35 mg 100 g^{-1} DW. This can be clarified by the fact that vitamin C is very sensitive to rubbing, temperature changes, and drying. Analyzing the fermented leaves, the highest quantity of vitamin C was after 24 h aerobic SPF (404.32 mg 100 g^{-1} DW) and after 24 h anaerobic SPF (357.16 mg per 100 g^{-1} DW). The highest amounts of dehydroascorbic acid were determined after 24 h aerobic SPF at 200.11 mg 100 g^{-1} DW and after 24 h and 72 h anaerobic SPF at 189.69 mg 100 g^{-1} DW and 193.39 mg 100 g^{-1} DW, respectively. L-ascorbic acid was mainly found in the control (unfermented leaves) at a concentration of 496.44 mg 100 g^{-1} DW, which is significantly higher compared to leaves fermented in all experiment variants (Table 1).

Table 1. The effect of solid-phase fermentation on the content of vitamin C, dehydroascorbic, and L-ascorbic acids in willowherb leaves (mg 100 g^{-1} DW).

Fermentation Method	Fermentation Duration	Vitamin C	Dehydroascorbic Acid	L-ascorbic Acid
			mg 100 g^{-1} DW	
Control (unfermented leaves)		678.35 ± 11.16 a [1]	179.91 ± 12.96 a	496.44 ± 21.61 a
Aerobic	24 h	404.32 ± 30.07 b	200.11 ± 39.30 a	204.20 ± 2.32 b
Aerobic	48 h	268.47 ± 9.55 de	135.67 ± 12.37 b	132.80 ± 19.38 d
Aerobic	72 h	241.35 ± 5.91 e	135.45 ± 1.56 b	105.89 ± 4.53 e
Anaerobic	24 h	357.16 ± 19.14 c	189.69 ± 7.52 a	167.47 ± 14.54 c
Anaerobic	48 h	287.18 ± 22.80 d	135.31 ± 28.14 b	151.87 ± 5.72 cd
Anaerobic	72 h	342.80 ± 5.33 c	193.39 ± 1.60 a	149.41 ± 4.97 cd
p-values (fermentation method × fermentation duration)		<0.0001	<0.0039	<0.0003

[1] The differences between the averages in columns marked by different small letters (a, b, c, d, e) are significant at $p < 0.05$.

The changes of the content of total chlorophyll, and two pigments of chlorophyll *a* and chlorophyll *b* in leaves during the fermentation process was estimated. The biggest amount of total chlorophyll in the leaves was determined after 72 h anaerobic SPF (610.03 mg 100 g^{-1} DW) and the lowest after 24 h aerobic SPF (379.16 mg 100 g^{-1} DW). The highest amount of chlorophyll *a* was determined in the leaves after 72 h anaerobic SPF (503.50 mg 100 g^{-1} DW) and the lowest after 24 h aerobic SPF (164.49 mg 100 g^{-1} DW), which corresponds to the trend of total chlorophyll content. The highest content of pigment of chlorophyll *b* was established in the control (unfermented leaves) (254.23 mg 100 g^{-1} DW) and the lowest after 72 h fermentation under aerobic and anaerobic conditions, respectively: 106.49 mg 100 g^{-1} DW and 106.53 mg 100 g^{-1} DW. This is the opposite trend to chlorophyll *a* and total chlorophyll contents (Table 2).

Table 2. The effect of solid-phase fermentation on the content of total chlorophyll, chlorophyll *a* and chlorophyll *b* in willowherb leaves (mg 100 g^{-1} DW).

Fermentation Method	Fermentation Duration	Total Chlorophyll	Chlorophyll *a*	Chlorophyll *b*
			mg 100 g^{-1} DW	
Control (unfermented leaves)		474.52 ± 32.80 b [1]	220.29 ± 0.55 d	254.23 ± 32.45 a
Aerobic	24 h	379.16 ± 28.30 c	164.49 ± 1.58 e	214.66 ± 27.83 a
Aerobic	48 h	472.33 ± 26.15 b	364.91 ± 29.56 b	107.42 ± 3.65 b
Aerobic	72 h	449.48 ± 5.30 b	342.99 ± 5.67 c	106.49 ± 3.04 b
Anaerobic	24 h	448.87 ± 68.93 b	340.32 ± 3.98 c	108.55 ± 65.04 b
Anaerobic	48 h	447.38 ± 4.62 b	334.86 ± 4.86 c	112.50 ± 1.61 b
Anaerobic	72 h	610.03 ± 2.77 a	503.50 ± 3.26 a	106.53 ± 3.96 b
p-values (fermentation method × fermentation duration)		<0.0008	<0.0001	<0.0087

[1] The differences between the averages in columns marked by different small letters (a, b, c, d, e) are significant at $p < 0.05$.

The highest content of total sugars in willowherb leaves was after 72 h of anaerobic SPF (8.06 mg 100 g^{-1} DW) and the lowest after 72 h of aerobic SPF (2.79 mg 100 g^{-1} DW). It was established that the highest amounts of fructose (2.66 mg 100 g^{-1} DW) and glucose (3.58 mg 100 g^{-1} DW) were identified after 72 h of anaerobic SPF and the lowest amounts after 48 h of aerobic fermentation (0.75 mg 100 g^{-1} DW and 0.83 mg 100 g^{-1} DW, respectively). The highest quantities of sucrose were found in unfermented leaves (2.62 mg 100 g^{-1} DW) and the lowest quantities after 72 h aerobic fermentation (1.21 mg 100 g^{-1} DW) (Table 3).

Table 3. The effect of solid-phase fermentation on the content of sugars in willowherb leaves (mg 100 g^{-1} DW).

Fermentation Method	Fermentation Duration	Total Sugars	Fructose	Glucose	Sucrose
		g 100 g^{-1} DW			
Control (unfermented leaves)		5.30 ± 0.03 c [1]	1.42 ± 0.04 e	1.26 ± 0.02 d	2.62 ± 0.06 a
Aerobic	24 h	5.53 ± 0.21 c	1.75 ± 0.09 c	2.18 ± 0.11 c	1.59 ± 0.05 c
Aerobic	48 h	3.03 ± 0.04 d	0.83 ± 0.00 f	0.86 ± 0.00 e	1.34 ± 0.04 d
Aerobic	72 h	2.79 ± 0.04 d	0.75 ± 0.00 f	0.83 ± 0.00 e	1.21 ± 0.04 e
Anaerobic	24 h	6.24 ± 0.08 b	2.15 ± 0.02 b	2.78 ± 0.05 b	1.32 ± 0.04 d
Anaerobic	48 h	5.46 ± 0.07 c	1.59 ± 0.02 d	2.10 ± 0.04 c	1.77 ± 0.09 b
Anaerobic	72 h	8.06 ± 0.27 a	2.66 ± 0.10 a	3.58 ± 0.10 a	1.82 ± 0.07 b
p-values (fermentation method × fermentation duration)		<0.0001	<0.0001	<0.0001	<0.0001

[1] The differences between the averages in columns marked by different small letters (a, b, c, d, e, f) are significant at $p < 0.05$.

3.2. Principal Component Analysis

Principal component analysis (PCA) was done to estimate the relationships between the different fermentation conditions (methods, as well as duration) and 10 parameters listed in Tables 1–3. The results of PCA are shown in Figure 1. The PCA indicated that the first two components explained 44.26% (PC1) and 39.35% (PC2) of the total variance (Figure 1). Eigenvalues of the PC1 and PC2 were higher than one (4.43 and 3.94, respectively). Vitamin C, dehydroascorbic acid, L-ascorbic acid, chlorophyll *b*, and sucrose were highly positively associated with the first factor (PC1). The second factor (PC2) was positively associated with chlorophyll (total), chlorophyll *a*, sugars (total), glucose, and fructose.

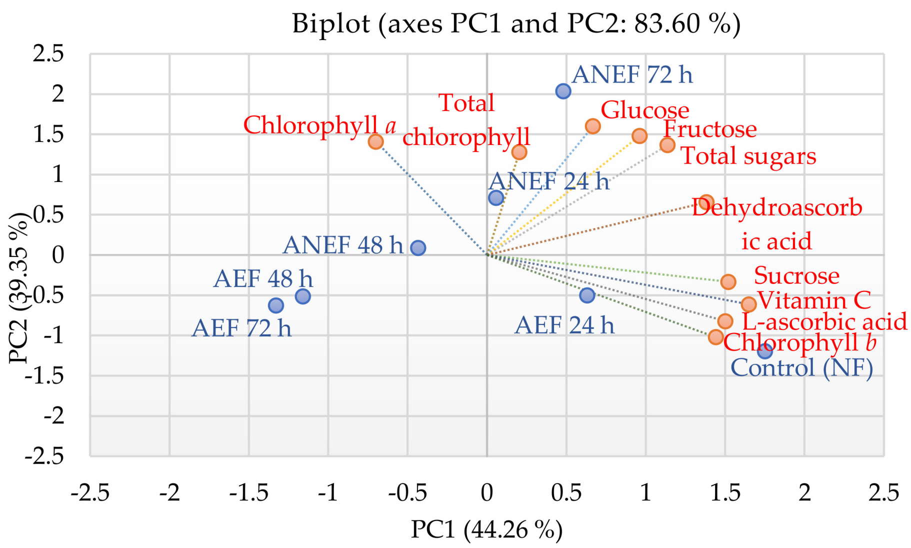

Figure 1. Principal component analysis of vitamin C, dehydroascorbic acid, L-ascorbic acid, total chlorophyll, chlorophyll *a*, chlorophyll *b*, total sugars, glucose, fructose, and sucrose in willowherb leaves (NF (control)—unfermented leaves (control), AEF 24 h—aerobic fermentation 24 h, AEF 48 h—aerobic fermentation 48 h, AEF 72 h—aerobic fermentation 72 h, ANEF 24 h—anaerobic fermentation 24 h, ANEF 48 h—anaerobic fermentation 48 h, ANEF 72 h—anaerobic fermentation 72 h).

As shown in Figure 1, the NF (control) and AEF 24 h were associated with higher amounts of vitamin C, L-ascorbic acid, chlorophyll *b*, and sucrose. ANEF 24 h and ANEF 72 h were grouped closely due to the higher amounts of sugars (total), fructose, glucose, chlorophyll (total), and chlorophyll *a*.

4. Discussion

During the solid-phase fermentation of willowherb leaves, various chemical reactions occur, during which complex compounds are broken down into simpler ones, some substances are transformed into others, and thus both qualitative and quantitative changes occur [9–11].

Vitamin C is necessary for human health. Many diseases, such as infections, cancer, cardiovascular disease, stroke, diabetes, and sepsis, have been associated with poor vitamin C levels [12,13]. Decreased vitamin C levels in disease are often explained by a combination of a sometimes massively increased turnover due to oxidative stress and inflammation and a decreased dietary intake of vitamin C associated with the disease [14,15].

From the presented data (Table 1) we can see that solid-phase fermentation significantly decreased the amounts of vitamin C in its oxidized form, dehydroascorbic acid, and L-ascorbic acid. Vitamin C highest content was found in unfermented willowherb leaves (678.35 mg 100 g^{-1} DW). The vitamin C present in the leaves of willowherb is very sensitive to rubbing, softening, changes in temperature, oxygen, and the method of drying. Therefore, there were significantly higher amounts of vitamin C in the raw material of willowherb leaves than in fermented ones. As we can see from the obtained data (Table 1), the decrease in vitamin C content was most significantly influenced by the duration of fermentation: the longer the duration of the fermentation the less vitamin C remained in the leaves. For example, after 24 h aerobic SPF, vitamin C was 404.32 mg 100 g^{-1} DW, after 48 h aerobic SPF decreased by 33.60% (to 268.47 mg 100g^{-1} DW), and after 72 h aerobic SPF decreased by 40.31% (to 241.35 mg 100 g^{-1} DW) compared to the control (678.35 mg 100 g^{-1} DW).

Chlorophylls fulfill various vital functions in plants. In the chloroplast, chlorophylls are the predominant light-absorbing pigment in photosynthesis [16]. The dominant pigment in pomelo leaves, chlorophyll, has been reported to possess many biological activities such as antioxidant, anti-inflammatory, and anticancer properties [17].

The ratio of total chlorophyll *a* and chlorophyll *b* in the control (unfermented leaves of willowherb) was almost the same, but chlorophyll *a* increased significantly after fermentation. This difference was most pronounced after 72 h of anaerobic fermentation (chlorophyll *a*: 503.50 mg 100 g^{-1} DW and chlorophyll *b*: 106.53 mg 100 g^{-1} DW). This could be seen by the variation of intensity of the colors: in the control, the leaves were bright green, and during fermentation this color turned more and more brownish green until it turned almost brown after 72 h of fermentation. Moreover, during fermentation, chlorophyll compounds and other pigments undergo degradation due to the accumulation of lactic acid and the resultant decline in pH [18].

Sugars, organic acids, and amino acids are the major energy sources. It is produced via photosynthesis and respiration in plants [19]. During solid-phase fermentation, one of the steps is the hydrolysis of primary polymeric substances to finer substances (e.g., disaccharides (sucrose) to monosaccharides (fructose and glucose)). From the presented data we can see that during aerobic fermentation both the total amounts of sugars and the amounts of fructose, glucose, and sucrose decreased significantly. However, during anaerobic fermentation, total sugars, fructose, and glucose increased, but sucrose decreased. This could be explained by the fact that under solid-phase fermentation the decomposition of disaccharide sucrose into monosaccharides occurs. In this case fructose and glucose occurs more intensively under anaerobic conditions than during aerobic fermentation.

5. Conclusions

The discussed results showed that SPF affected the amounts of biologically active substances in the leaves of willowherb. Vitamin C levels were significantly reduced in all variants, so it would be appropriate to recommend non-fermented willowherb leaves (control variant) as a plant source of vitamin C.

Total chlorophylls content increased significantly after 72 h of anaerobic fermentation, and chlorophyll *a* levels were significantly higher in all variants (except after 24 h of aerobic fermentation) compared to control. Therefore, according to these data, it would be possible to recommend willowherb leaves after 72 h of anaerobic SPF as a source of chlorophylls (especially chlorophyll *a*).

The highest quantities of sugars were found after anaerobic SPF. The sugars increased significantly after 72 h of anaerobic fSPF, compared to unfermented willowherb leaves.

In summary, we can say that SPF activates both the degradation of cell walls and the activity of microorganisms and enzymes, which leads to quantitative and qualitative changes in biologically active substances. Based on the data of this study, 72 h of anaerobic SPF could be suggested for the food and pharmacy industry to produce willowherb leaf functional food products with higher chlorophylls and sugar contents, and unfermented leaves of willowherb could be very useful for people due to high vitamin C content.

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