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Application of Response Surface Methodology for Fermented Plant Extract from *Syzygium aromaticum* L. (*Myrtaceae*): Optimisation of Antioxidant Activity, Total Polyphenol Content, and Lactic Acid Efficiency

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Abstract: *Syzygium aromaticum* L. *Myrtaceae* is one of the plants rich in bioactive compounds that have beneficial effects on the skin. Fermented plant extracts (FPEs) obtained from this plant have recently been exploited as new cosmetic ingredients. The concept of our study was related to the use of clove buds in the fermentation process in order to obtain new cosmetic raw materials with high antioxidant potential. The focus was on evaluating antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA_e). For this purpose, the most favourable technological parameters of the fermentation process of clove buds were determined, including the type of microorganisms, initial sugar content, plant raw material content, and fermentation time. The most favourable parameters were correlated with the optimal parameters, which were determined based on response surface methodology (RSM). Based on DPPH and Folin–Ciocalteu assays and GC-MS analysis, optimal points of antioxidant activity against reactive oxygen species (analysed with RSM) were successfully selected, enabling quantitative mathematical representations. The optimisation revealed that using a strain of *Lactobacillus rhamnosus* MI-0272 in lactic fermentation and plant material (6.40%) and beet molasses (3.20%) results in the highest antioxidant potential of FPE (33.90 mmol Tx/L) and yielding LA with the highest efficiency (96%). The optimised FPE had higher polyphenol content (11.60 mmol GA/L ± 0.14), chelating and antioxidant activity (0.32 mmol Fe^{2+}/L ± 0.01 and 11.60 mmol Tx/L ± 0.09), and Fe^{3+} ion reduction (49.09 mmol Fe^{3+}/L ± 0.16) than the PE. In addition, the possibility of using the spent plant material remaining after the extraction process to prepare activated carbons capable of treating wastewater was investigated.

Keywords: mathematical modelling; fermented *Syzygium aromaticum* L. *Myrtaceae*; antioxidant activity; cytotoxicity assessment; adsorbent modifications

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1. Introduction

Plant extracts (PEs) have enormous potential and so might be important cosmetic raw ingredients for use in cosmetic formulations. Obtaining PE consists of the extraction of bioactive compounds contained in the plant material using methods such as maceration, solvent extraction, supercritical fluid extraction, or Soxhlet extraction [1]. Recently, fermented plant extracts (FPEs), characterised by antioxidant, anti-ageing, moisturising, and

anti-allergenic properties as well as enhanced bioactivity, biocompatibility, and bioavailability, have been used as innovative cosmetic raw materials [2–6]. Obtaining FPEs involves not only separating the desired bioactive compounds from the plant material but also decomposing or converting unwanted substrata into compatible products under the action of appropriate microorganisms. Microorganisms decompose plant components contained in plant materials, increasing the biological activity of the substrate by converting high-molecular compounds into low-molecular structures. The structural breakdown of the cell walls of plant raw materials and the hydrolyzing activity of microorganisms during the ongoing fermentation increase the content of anthocyanins, flavonoids, organic acids, proteins, ceramides, amino acids, or biological enzymes [7]. Bacteria and fungi may produce antioxidants during fermentation by enzymatically converting phenolic glycosides found in plant source materials into free polyphenols [4–7].

Polyphenolic compounds have piqued the interest of the beauty industry due to the multiple health advantages associated with their antioxidant and anti-inflammatory properties [2,8,9]. Polyphenols scavenge free radicals, neutralising their detrimental effects and decreasing oxidative stress in the body [1,8]. They also reduce risk factors for chronic illnesses such as cardiovascular disease and certain malignancies. Blueberry fruit fermentation in the presence of lactic acid bacteria (LAB) *Lactiplantibacillus plantarum* and *L. fermentum* indicates the existence of phenolic components in FPEs [10,11]. The fermented plant extract produced from *Magnolia denudata* flowers in the presence of *Pediococcus acidilactici* KCCM 11614 showed stronger anticancer activity than the unfermented plant extract against human gastric adenocarcinoma (AGS) cells and human colon cancer (LoVo) cells [12]. Plant extracts obtained by fermentation of *Rhus verniciflua* bark have shown anticancer efficacy against the colon cancer cell line HCT-116, as well as the potential to trigger apoptosis and block the hedgehog pathway [13,14]. Furthermore, the fermented plant extract produced from ginseng in the presence of *Aspergillus usamii* demonstrated stronger anticancer activity against human hepatoma cells (HepG2) and the human colon cancer cell line (DLD-1) than the unfermented ginseng extract [9,15,16].

Lactic acid (LA) is a metabolite produced by lactic acid bacteria in the presence of carbohydrates, more specifically sugars composed of six-carbon residues (glucose or sucrose) [17]. The most desirable in industrial production are the enantiomers L(+) of LA, which are produced mainly by the homofermentative lactic fermentation bacteria of the genus *Lactobacillus* [18]. Because of its powerful preservation effect, lactic acid has been employed in the processing of meat, fruit, and vegetables but has lately found usage in the cosmetic sector [19]. It is an authorised substance for use in food and is labelled E-270. (Dz. U. No. 232, Item 1525).

Several statistical methods of experimental design are currently used to optimise bio-processes. Response surface methodology (RSM) is one of the most widely used and appropriate methods for identifying the influence of individual variables and efficiently searching for optimal conditions for a multivariate system [20,21]. Response surface methodology is an experimental technique used in the analysis and optimisation of a given process in order to determine optimal process parameters. RSM describes the relationship between the experimental factors studied and the process response (for example, the efficiency of the main product or the antioxidant activity of the fermented plant extract), where the response surface is approximated by mathematical functions (for example, polynomials) that allow prediction of response values for different combinations of factors. The RSM technique offers numerous advantages for optimising the fermentation process to obtain fermented plant extracts (FPEs) with the highest values of the process parameters tested while reducing the number of experiments required [22,23].

Syzygium aromaticum L. belongs to the *Myrtaceae* family, which includes more than 3000 species and about 150 genera, such as the myrtle, eucalyptus, clove, and guava families. Clove essential oil is obtained by distilling the flowers, stems, and leaves of *Syzygium aromaticum* L. *Myrtaceae*. Clove comprises approximately 15–20% of CEO, which contains a high amount of phenolic compounds with several biological activities, including

antibacterial, antifungal, insecticidal, and antioxidant properties. At least 30 chemicals have been discovered in CEO, with eugenol accounting for at least 50%. The remaining 10–40% contains eugenyl acetate, β-caryophyllene, and α-humulene. Minor or trace components (less than 10%) include diethyl phthalate, caryophyllene oxide, cadinene, α-copaene, 4-(2-propenyl)-phenol, chavicol, and α-cubebene. The Food and Drug Administration (FDA) classifies CEO as generally recognised as safe (GRAS); for this reason, it is used in perfumes, cosmetics, sanitary products, medicines, and foods. CEO was recently recognised as an effective anaesthetic for sedating fish. This essential oil has also shown anti-inflammatory effects in meat and cheese. Due to its properties, the oil finds numerous applications, especially in the pharmaceutical industry (as an ingredient in warming and analgesic ointments) and in dentistry (as an ingredient in analgesic preparations, tooth cavity fillings, mouth and gum rinses, and toothpastes). Cosmetic formulas for acne skin care recommend eugenol because it improves the complexion of people with acne and psoriasis, reducing the appearance of lesions and accelerating their healing. Eugenol, a pale yellow liquid, is classified as an absorption promoter, which is characterised by high antimicrobial activity as well as antioxidant activity. It is a very interesting compound, considering not only its applications in medicine (an antiseptic in dental and medical practice) and cosmetics but also in organic synthesis and polymerisation processes (for example, it is used as an antioxidant for plastics and rubber). In addition, eugenol is widely used as a flavouring agent in food, beverages, candy, and frozen products. It is also present in many other aromatic plants, such as basil, cinnamon, and bay leaves [24–30].

In our previous study, the non-fermented and fermented extracts were obtained from the ground and defatted seeds of spotted thistle *Silybum marianum*. Their antioxidant activity was evaluated using DPPH, ABTS, and FRAP techniques, while total polyphenol content was measured using the Folin–Ciocalteu method. High antioxidant activity was found for both the extract (0.91 mmol Trolox/L $\pm$ 0.2) and the bio-ferment (1.19 mmol Trolox/L $\pm$ 0.2), which was evaluated by the DPPH technique, so the resulting cosmetic raw materials were incorporated into hydrogel (H) and organogel (O) vehicles to obtain cosmetic formulations with antioxidant activity. We then evaluated the in vitro permeation through porcine skin of the main components contained in the obtained cosmetic raw materials, such as silibinin and taxifolin, which are part of the silymarin complex. For comparison, we also used pure silymarin (S). Of the formulations tested, H-S showed the most significant penetration of taxifolin, with a cumulative permeation of 87.739 $\pm$ 7.457 $\mu g/cm^2$. Finally, biodegradation tests of prepared formulations containing cosmetic raw materials and silymarin were also conducted. Tests on the effect of cosmetic formulations on aerobic biodegradation showed a good level of degradation of the prepared formulations, some of which (O-B and O-S) were classified as readily degradable (OECD) [3].

Recently, there has been an increase in interest in using plants and plant-based materials as potential ingredients in topical medicinal and cosmetic products. Increased environmental awareness among consumers, as well as the widespread belief that plant-based remedies are healthier than those derived from synthetic ingredients, are driving this interest. One such interesting plant seems to be *S. aromaticum* L. *Myrtaceae*, which is used only as an ingredient in cosmetic formulations in the form of clove essential oil or eugenol [31–33]. However, despite its health-promoting potential, the popularity of fermented extracts from this plant is low. In particular, the possibility of its use in cosmetic preparations for topical use is underestimated. There are no reports in the available literature regarding the fermented plant extract (FPE) obtained from *S. aromaticum* L. *Myrtaceae*. There is also no information on optimising the fermentation process of clove buds using response surface methodology (RSM). In our article, we propose the possibility of using clove buds to obtain fermented plant extracts with a much higher content of phenolic compounds and higher antioxidant activity than PE from buds. For this purpose, a study of the influence of technological parameters on the fermentation process of clove buds was conducted. During the study of this process, the influence of such technological pa-

rameters as the type of microorganism, initial sugar content, plant raw material content, and fermentation time was studied in detail. The quantities describing the process were antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA). Particular attention was paid to determining the process parameters at which the main fermentation product (LA) was obtained with the highest efficiency, with simultaneously high values of AA and TPC. Moreover, in this study, we will use response surface methodology (RSM) for the first time in the fermentation of clove buds in the presence of lactic acid bacteria and using molasses, a by-product of the sugar industry. The fermentation process of plant raw materials has been relatively well understood and described in our previous publication [3], but there is still a lack of information on how to optimise the fermentation process to determine optimal process conditions. The study presented here aims to evaluate the influence of process parameters on the antioxidant activity (AA) of fermented plant extracts, the content of phenolic compounds (TPC), and the efficiency of lactic acid (LA). Conducting this study will enable optimisation of the process and the selection of parameters at which the obtained cosmetic raw materials will have the richest chemical composition. In addition, beet molasses, which is a by-product obtained in the production of sucrose from sugar beet, is a relatively cheap, readily available raw material and can be used to produce lactic acid during fermentation (eliminating the need for sugar). After the optimisation process, the chelating (Fe^{2+} ions) and reducing (Fe^{3+} to Fe^{2+} ions) properties were assessed, and the cytotoxicity of the optimised FPE was examined. We used the same methods to evaluate the clove buds extract for comparison. In addition, the possibility of using the spent plant material remaining after the extraction process to prepare activated carbons capable of treating wastewater was investigated.

2. Materials and Methods

2.1. Materials

We purchased ground clove buds (*S. aromaticum* L. *Myrtaceae*) for optimisation studies from the herbal store Aromatika Adam Iwaczuk (Hajnowka, Poland). The average granularity of the raw materials was less than 250 µm. We obtained the beet molasses from "Cukrownia Kluczewo" (Stargard, Poland). The molasses contained 80% of the total amount of 6-carbon sugars (Brix). DPPH (2,2-diphenyl-1-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Tx) were acquired from Sigma Aldrich (Sigma-Aldrich Merck Group, St. Louis, MO, USA). Folin–Ciocalteu reagent, iron II sulfate heptahydrate, iron II sulfate VI, ferrozine, iron III chloride, gallic acid, the amino acid L-glutamine, FBS (Fetal Bovine Serum), penicillin, streptomycin, resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt), and Neutral Red dye were obtained from Merck, Darmstadt (Germany). We purchased a medium for lactic acid bacteria (CM0359) from OXOID (M.R.S. BROTH, Rogosa, Sharpe). Probiotical (Novara, Italy) provided the strains of lactic acid bacteria: *Lactobacillus salivarius* LY_0652; *Lactobacillus reuteri* MI_0168; *Lactobacillus acidophilus* MI-0078; *Lactobacillus brevis* LY_1120; *Lactobacillus plantarum* MI-0102; and *Lactobacillus rhamnosus* MI-0272. Lipase AY30 was acquired from Thermo Scientific (Białystok, Poland). Chempur (Piekary, Poland) delivered octane, methanol, ethanol (96%), methylene blue ($C_{16}H_{18}ClN_3S$, MB), potassium hydroxide (KOH), sulfuric acid VI, and phosphate-buffered saline PBS (pH 7.40 $\pm$ 0.05). We purchased human fibroblasts (HDF) and keratinocytes (HaCaT) from CLS Cell Lines Service (Eppelheim, Germany). We obtained DMEM medium from Dulbecco's Modified Essential Medium, Biological Industries, located in Kibbutz Beit-HaEmek, Israel. All reagents were of analytical grade.

2.2. Preparations of Fermented Plant Extract (FPE) and Plant Extract (PE)

2.2.1. Preparations of FPE

New fermented plant extracts (FPEs) were obtained by the fermentation of clove buds. In our study, as a raw material to produce lactic acid, we used beet molasses, which is a waste product of the sugar industry. We determined the total content of 6-carbon sugars

(Brix) present in the molasses using a refractometer method (KRUSS Optronic DR301-95, A. Kruss Optronic GmbH, Hamburg, Germany). The buds fermentation process used beet molasses, which eliminated the need for additional mineral salts in the form of nitrates or ammonium salts, chlorides, and potassium phosphate [34]. Preliminary tests verified that the nitrogen and phosphorus contained in molasses were sufficient for proper fermentation. For this purpose, molasses aqueous solutions were fermented with 3.20% mixed inoculum (the amount of which was determined based on our previous studies), but without the presence of mineral salts [3]. Preliminary studies have shown that beet molasses containing up to 2.0% total nitrogen has sufficient amounts of essential nitrogen to produce lactic acid [24]. This nitrogen is mainly represented by amino acids such as glutamic acid, aspartic acid, and alanine, and, to a small extent, also by ammonium salts. The phosphorus content (about 0.2%) in molasses also proved sufficient for fermentation [29,30]. Adequate growth of lactic acid was observed in all molasses solutions, to a similar extent to fermentations carried out in the presence of certified cane molasses requiring the additional use of mineral salts such as ammonium sulphate (0.64%), calcium chloride, and potassium dihydrogen phosphate (0.32%) [3].

Next, the fermentation of the buds was carried out using lactic acid bacteria. The fermentation was carried out until the maximum level of LA was reached, the concentration of which was controlled by the GC-MS method. The level of total polyphenol content (TPC) was also monitored during fermentation. In addition to the matrix of the plant material used, the bioavailability of phenolic compounds is also affected by the type of LAB strain used. Therefore, fermentation of buds was carried out using 6 individual strains of LAB: *L. reuteri* MI_0168, *L. salivarius* LY_0652, *L. brevis* LY_1120, *L. acidophilus* MI-0078, *L. rhamnosus* MI-0272, and *L. plantarum* MI-0102.

We prepared the inoculum according to a previously used procedure [3]. The subsequent raw materials were presented into a 500 mL conical flask: beet molasses (the amount of which was controlled depending on the process parameter under study while maintaining an initial sugar content of 1.0–5.0%), distilled water, plant material constituting clove buds (the amount of which was controlled depending on the process parameter under study while maintaining a content of 1.0–7.0%), and inoculum (in an amount of 3.20%). The content of inoculum in the fermentation medium was determined earlier, as a result of preliminary studies [3]. The contents of the flask were agitated until the molasses was completely dissolved; subsequently, the fermentation process commenced (at a temperature of 37.5 °C) for an appropriate time. During the fermentation process, samples were collected and examined for lactic acid content (whose concentration was determined by GC-MS) and polyphenols (whose levels were determined by the spectrophotometric method using the Folin–Ciocalteu technique). The fermentation was carried out until the maximum level of lactic acid was reached. The highest content of LA was observed on the 9–10 days (depending on the process parameter under study) of fermentation, and continuing the process had the effect of decreasing LA concentration.

Two independent experiments carried out the fermentation process. After the process was completed, lipase was added to hydrolyze the bacterial cell wall, and the obtained fermented plant extracts were subjected to 3-stage filtration: 1. initially, the FPE underwent filtration using a glass funnel, 2. subsequently, the FPE underwent centrifugation using a centrifuge (5 min, 166 Hz, 10,000 g), and 3. ultimately, the FPE that had undergone extra filtration and centrifugation was further filtered using sterile syringe filters with a pore size of 0.45 µm (intended for sterilising filtration of aqueous solutions). In this way, the fermented plant extract was free of microorganisms [3]. The amount of extract obtained and filtered was approximately 110–120 mL. The FPE was kept in a freezer at a temperature of −15 °C.

2.2.2. Preparations of PE

The water extract was prepared by ultrasound-assisted extraction (40 kHz) using an ultrasonic bath with a thermostat (FSF-031S, ChemLand, Stargard, Poland). For this

purpose, 22 g of ground plant material (in the amount of 6.40%) was introduced into 300 mL of distilled water, and extraction was carried out for 20 min (40 ± 1.0 °C), after which the extract obtained was filtered on a pressure funnel through a Whatman paper filter (EEA03).

2.3. Total Polyphenols Content (TPC)

The Folin–Ciocalteu method was employed to determine the total polyphenols content of FPEs [31]. These studies were conducted using the Thermo Scientific GENESYS 50 instrument (Waltham, MA, USA) at the wavelength λ = 750 nm. Gallic acid (GA) was used as a reference substance. TPC was expressed as mmol GA/L of FPE based on the resulting calibration curve of gallic acid (y = 0.0075x, R^2 = 0.997).

We quantified the total polyphenol content of the FPEs as follows: 2000 µL of Folin–Ciocalteu reagent, 100 µL of appropriate extract, and 1000 µL of aqueous Na_2CO_3 (saturated solution) were introduced into 10 mL flasks. The contents of the flasks were made up to the mark with distilled water, the flasks were closed tightly with a stopper and incubated at an ambient temperature for a duration of 15 min, after which the absorbance of the test solutions was measured using a spectrophotometer at a wavelength of λ = 750 nm. Blank samples of the absent FPE were prepared in the same way, using distilled water in an amount of 100 µL. Three independent experiments were performed.

The total polyphenols (*TPC*) content was determined using the following Formula (1):

$$TPC = \frac{[(C_{FAt.s.} - C_{FAb.s.}) \cdot V_s]}{V_{FPE}} \cdot 100\% \quad (1)$$

where

TPC—Total polyphenol content by the F-C method [mmol/L],
$C_{FAt.s.}$—concentration of phenolic acids in tested sample [mmol/L],
$C_{FAr.b.}$—concentration of phenolic acids in the blank sample [mmol/L],
V_s—total volume of solution introduced into volumetric flasks [L],
V_{FPE}—volume of FPE introduced into volumetric flasks [L].

2.4. Antioxidant Activity (DPPH Free Radical Method)

The DPPH free radical method was employed to assess the antioxidant capability of the acquired FPEs [31]. These studies were conducted using the Thermo Scientific GENESYS 50 instrument at the wavelength λ = 517 nm. Trolox (Tx) was used as a reference substance.

The antioxidant activity of the FPEs was measured as follows: 2850 µL ethanolic solution of the DPPH radical (the concentration of 0.3 mmol/L) of absorbance about 1.000 ± 0.020 (at λ = 517 nm) was placed in the tube, and 150 µL of appropriate extract was added. Blank samples without FPE were prepared in the same way, using distilled water in an amount of 150 µL. The tubes were enveloped in aluminum foil, sealed with a stopper, and then incubated for 10 min at ambient temperature. Three independent experiments were carried out, with each sample analysed being diluted 50-fold before the assay was performed. The antioxidant activity was expressed in mmol Tx/L FPE, based on the obtained calibration curve y = −1.0321x + 1.1342, R^2 = 0.997.

The antioxidant activity (*AA*) of fermented plant extract was determined using the following Formula (2):

$$AA = -0.019\,(A_{t.s.} - A_{b.s.}) + 1.1342 \quad (2)$$

where

AA—antioxidant activity by DPPH method [mmol Tx/L],
$A_{t.s.}$—absorbance of the tested sample [-],
$A_{b.s.}$—absorbance of the blank sample [-].

2.5. GC-MS Analysis

The gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Shimadzu GCMS-QP2020 NX (Shimadzu, Kyoto, Japan) with a Shimadzu SH-I-5MS column (30 m × 0.25 mm × 0.25 µm). The column temperature was kept at 40 °C for 2 min and programmed to 280 °C at a rate of 15 °C/min. The flow rate of helium as a carrier gas is 35 cm/s (1 µL/min). MS were taken at 70 eV, using split 10. The total analysis time was 17 min, while the volume of sample dispensed was 1 µL. Identification of lactic acid formed during the fermentation process was made by comparison of mass spectra located in the spectra library (NIST2020) with the LA benchmark used.

The concentration of lactic acid (C_{LA}) in fermented plant extracts was calculated based on the obtained calibration curve (R^2 = 0.9971) using the internal standard method: octane (3):

$$C_{LA} = (1.518 \times S_{LA} + 1568) / S_O \quad (3)$$

where

A—slope,
B—intercept,
C_{LA}—lactic acid concentration [%],
S_{LA} and S_O—lactic acid and octane peak area.

All samples were analysed three times. Results are presented as the mean ± standard deviation (SD).

2.6. Fermentation of Clove Buds in the Presence of Lactic Acid Bacteria

A study of the influence of technological parameters on the fermentation process of clove buds was conducted. The influence of the following process parameters was studied: type of microorganism, initial sugar content, plant raw material content, and fermentation time (Table 1). The most favourable technological parameters were selected based on the values of the main functions describing the process, such as antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA_e).

Table 1. Technological parameters affecting the fermentation process of clove buds and the ranges of their changes.

Parameters of the Fermentation Process	Unit	Ranges of Change
Type of microorganism	-	*L. reuteri* MI_0168 *L. salivarius* LY_0652 *L. brevis* LY_1120 *L. acidophilus* MI-0078 *L. rhamnosus* MI-0272 *L. plantarum* MI-0102
Initial sugar content	%	1.60–4.60
Plant raw material content	%	0.32–6.40
Fermentation time	days	1–11

2.7. Response Surface Method for Fermentation Process Optimisation

Mathematical optimisation of the fermentation process of clove buds using beet molasses (whose total content of 6-carbon sugars was 80%) in the presence of lactic acid bacteria was carried out according to the procedure described in Section 2.2.

The main functions describing the fermentation process were (1) antioxidant activity (AA) assessed by the DPPH method; (2) total phenolic compounds (TPC) assessed by the Folin–Ciocalteu method; and (3) lactic acid efficiency calculated on the basis of a calibration curve prepared by the internal standard method. Optimal process parameters were determined, that is, those at which maximum values of the main functions describing the process were obtained. Contour drawings were plotted for the three studied functions, and their analysis was carried out. During the optimisation of the fermentation process, a

central composition plan (CCD) was used. The experimental plan and contour drawings were made using the computer program Statistica 13.3.

The coefficients of the regression equations for the normalised input quantities were determined by the least squares method using matrix calculus. An approximating function was determined for each function, after which its adequacy was checked using the ANOVA test. In addition, multivariate correlation coefficients (R^2) were also calculated.

The effect of the normalised independent factors (X_i, X_j) of the fermentation process on the value of the response function (Y_i) is shown by a second-degree algebraic polynomial (4):

$$Y_i = a_0 + \sum_{i=1}^{3} a_{i1} \cdot X_i + \sum_{i=1}^{3} a_{i2} \cdot X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} \cdot X_i \cdot X_j \quad (4)$$

The waveforms of each function were plotted with simultaneous changes in two process parameters, while the other parameters were fixed and took values consistent with those determined for the maximum of each function.

In order to obtain the most adequate mathematical description of the course of the fermentation process of clove buds, three technological parameters (the content of plant raw material, the initial concentration of sugars, and the time of fermentation) were taken into account, the ranges of changes of which were narrowed down on the basis of the results obtained earlier during the research using the single variable method. The ranges of change in technological parameters were determined as follows: X_1 (plant raw material content): 1.0–7.0%, X_2 (initial sugar concentration): 1.0–5.0%, and X_3 (fermentation time): 1–9 days.

The actual values of the input quantities were used to carry out the calculations.

The PRESS values were determined based on Equation (5):

$$PRESS = \sum_{i=1}^{n} (Y_i - Y_{ie})^2 \quad (5)$$

where

Y_i—predicted value,

Y_{ie}—experimental value.

Table 2 shows the most relevant statistics for optimisation using response surface methodology (RSM): the determined coefficients of Equation (4) a_0, a_1, a_2, a_3, a_4, and a_5 for the function ranges tested, the correlation coefficients (R^2), the adjusted R-square ($adjR^2$), the standard deviation (SD), the relative standard deviation (RSD), and the PRESS value.

Table 2. The most favourable parameters for the fermentation process of clove buds in the presence of LAB and beet molasses and the corresponding values of the main functions of the process.

Fermentation Process Parameters	Unit	
Type of microorganism	-	*L. rhamnosus* MI-0272
Initial sugar content	%	3.20
Plant raw material content	%	6.40
Fermentation time	days	10
Functions of the fermentation process		
AA	mmol Tx/L	33.90 ± 0.04
TPC	mmol GA/L	11.60 ± 0.04
LA_e	%	96

2.8. Cytotoxicity Analysis

After fermentation and extraction of the plant raw materials, the resulting FPE and PE were evaporated to dryness. The dry matter content of the fermented plant extract was 5.5%, while that of the plant extract was 4.8%. The evaporated extracts were then diluted

with purified water at room temperature (approximately 20 °C) to the final concentrations and subjected to further analyses [35].

When assessing cytotoxicity, the following FPE concentrations were used: 0.1% (corresponding to 0.055 mg/mL); 1.0% (corresponding to 0.55 mg/mL); 2.5% (corresponding to 1.375 mg/mL); 5.0% (corresponding to 2.75 mg/mL); and 10.0% (corresponding to 5.5 mg/mL). In contrast, concentrations for PE were as follows: 0.1% (corresponding to 0.048 mg/mL); 1.0% (corresponding to 0.48 mg/mL); 2.5% (corresponding to 1.196 mg/mL); 5.0% (corresponding to 2.39 mg/mL); and 10.0% (corresponding to 4.8 mg/mL).

In the next stage, the cytotoxic properties of FPE and PE from *S. aromaticum* L. *Myrtaceae* were assessed. Analyses were performed on two cell lines: fibroblasts (HDF) and keratinocytes (HaCaT). The Neutral Red and Alamar Blue assays used in the study allowed the degree of cell growth inhibition to be assessed depending on the concentration of extracts tested. In the case of the Alamar Blue assay, resazurin, which undergoes a colorimetric change as a result of changes in cellular metabolism, was used as an oxidation-reduction indicator to assess cell viability in vitro. In contrast, viability and proliferative capacity (Neutral Red) were used to assess the proliferation of the cells studied. By measuring the amount of dye released, the total number of viable cells treated with the extracts was determined [36].

2.8.1. Cell Culture

Cytotoxicity analyses of the optimised FPE from *S. aromaticum* L. *Myrtaceae* were performed on human fibroblasts (HDF) and keratinocytes (HaCaT) obtained from CLS Cell Lines Service. Cells were grown in culture flasks in an incubator at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2). Both types of skin cells were cultured in DMEM medium (Dulbecco's Modified Essential Medium) with the addition of the amino acid L-glutamine, 10% (*v*/*v*) FBS (Fetal Bovine Serum), and 1% (*v*/*v*) antibiotics (100 U/mL penicillin and 1000 µg/mL streptomycin). Cytotoxicity analyses were performed on cells growing in 96-well plates at a density of 1×10^4 cells/well.

2.8.2. Alamar Blue (AB) Assay

The cytotoxicity of the optimised FPE from *S. aromaticum* L. *Myrtaceae* was assessed by measuring the ability of keratinocytes and fibroblasts to reduce resazurin after exposure to the tested samples. For this purpose, we used the methodology previously described by Page et al. [25] with minor modifications. Briefly, the tested cells were exposed to FPE dissolved in DMEM culture medium at concentrations of 0.1–10.0% for 24 h. After this time, the solutions were aspirated, and a resazurin solution with a concentration of 60 µM was added to each well and incubated for 2 h. Control cells were fibroblasts and keratinocytes separately, not exposed to FPE, and cultured in DMEM culture medium. After 2 h, fluorescence was measured in individual wells at λ = 570 nm using a Filter Max UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As part of the analyses, three independent experiments were carried out, in which each concentration of the analysed samples was tested in triplicate.

2.8.3. Neutral Red (NR) Uptake Assay

Cytotoxicity analyses were also performed using a test assessing the uptake of Neutral Red dye by lysosomes of keratinocytes and fibroblasts exposed to various concentrations of the tested FPE. The analysis was carried out based on the methodology previously described by Zagórska-Dziok et al. [26]. Briefly, cells seeded in 96-well plates were subjected to a 24-h incubation with the test samples dissolved in DMEM medium at concentrations of 0.1, 1.0, 2.5, 5.0, and 10.0%. Then, the samples were aspirated, and 40 µg/mL of Neutral Red dye (Merck KGaA, Darmstadt, Germany) was added to each well and incubated for 2 h. The dye was then aspirated, and the cells were washed with sterile PBS. Then, 150 µL of destaining buffer ($C_2H_5OH/CH_3COOH/H_2O$, 50%/1%/49%) was added to each well and shaken for 5 min on an orbital shaker. After this time, the absorbance at λ = 570 nm

was measured using a Filter Max UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As part of the research, three independent experiments were carried out on each sample tested in triplicate.

2.9. Reducing Fe^{3+} to Fe^{2+}

First, a calibration curve for Fe^{2+} ions was created using aqueous solutions of VI iron II sulphate ($FeSO_4$). To reduce any Fe^{3+} ions in the test sample, the following were added to 100 mL volumetric flasks: suitable quantities of $FeSO_4$ (such that the resultant concentrations of Fe^{2+} ions are in the range of 0.5 to 5 mg/L) and 1 mg of ascorbic acid. The flasks are filled to the mark with distilled water, then closed snugly with a stopper and agitated to produce uniform solutions [3].

First, 1000 µL of an aqueous $FeSO_4$ solution with ascorbic acid is put into a test tube. Then, 1000 µL of an aqueous ferrozine solution (with a concentration of 1 g/L) is added. The tubes are tightly sealed with a stopper and incubated at room temperature for 10 min, and then the absorbance of the test solutions is measured using a spectrophotometer at a wavelength of 562 nm. Spectrophotometric analyses are carried out in triplicate using a Thermo Scientific GENESYS 50 instrument, obtaining a calibration curve for Fe^{2+} ions ($y = 0.4888x + 0.0064$; $R^2 = 0.999$).

Assessment of the ability to reduce iron III ions to iron II by the ferrozine method was carried out as follows: 1000 µL of aqueous $FeCl_3$ solution (with an Fe^{3+} concentration of 0.5 g/L), 1 µL of FPE, and 1000 µL of aqueous ferrozine solution (with a concentration of 1 g/L) were introduced into glass tubes. The tubes were closed tightly with a stopper and incubated at room temperature for 10 min, and then the absorbance of the test solutions (λ = 562 nm) was measured using a spectrophotometer. First, the instrument was zeroed using 1000 µL of aqueous $FeCl_3$ solution, 1 µL of distilled water, and 1000 µL of aqueous ferrozine solution as a reference.

The reduction activity (RA) of Fe^{3+} to Fe^{2+} was estimated using the following Formula (6):

$$RA = \frac{[(C_{Fe2+r.s.} - C_{Fe2+t.s.}) \cdot V_s]}{V_{FPE}} \cdot 100\% \quad (6)$$

where

RA—reduction activity of Fe^{3+} [mg/L],
$C_{Fe2+r.s.}$—the concentration of Fe^{2+} ions in the reference sample [mg/L],
$C_{Fe2+t.s.}$—the concentration of Fe^{2+} ions in the tested sample [mg/L],
V_s—total volume of solution introduced into volumetric flasks [L],
V_{FPE}—volume of FPE introduced into volumetric flasks [L].

2.10. Chelating Activity of Fe^{2+} Ion

The chelating capacity of Fe^{2+} was evaluated using the ferrozine method [27]. The process of metal ion chelation significantly contributes to the prevention of reactive oxygen species formation [28]. A calibration curve was prepared by utilising aqueous solutions of $FeSO_4$ to measure Fe^{2+} ions. First, an initial $FeSO_4$ solution with an Fe^{2+} concentration of 0.53 mmol/L was prepared. Next, a ferrozine initial solution (concentration of 3.2 mmol/L). Then 1000 µL of the initial $FeSO_4$ solution (final Fe concentrations of 3; 1.2; 0.6; and 0.3 mg/L) and 1000 µL of ferrozine were introduced into 10; 25; 50; and 100 mL volumetric flasks. The flasks were filled with distilled water, sealed snugly with a stopper, and then kept at room temperature for a duration of 10 min. The absorbance of the test solutions was measured (using the Thermo Scientific GENESYS 50 apparatus at the wavelength λ = 562 nm), obtaining a calibration curve for Fe^{2+} ions $y = 0.4888x + 0.0064$, $R^2 = 0.999$.

In the next stage, 1000 µL of the initial $FeSO_4$ solution, 100 µL of FPE, and 1000 µL of ferrozine were introduced into 10 mL volumetric flasks. The flasks were filled with distilled water, sealed snugly with a stopper, and left to incubate at room temperature for 10 min, and then the absorbance of the solutions was measured using a spectrophotometer at a wavelength of λ = 562 nm. Three separate tests were conducted.

The chelating activity (*ChA*) of Fe^{2+} ions was calculated using the following Formula (7):

$$ChA = \frac{[(C_{Fe2+r.s.} - C_{Fe2+t.s.}) \cdot V_s]}{V_{FPE}} \cdot 100\% \quad (7)$$

where

ChA—chelating activity of Fe^{2+} [mg/L],
$C_{Fe2+r.s.}$—concentration of Fe^{2+} ions in the reference sample [mg/L],
$C_{Fe2+t.s.}$—concentration of Fe^{2+} ions in the tested sample [mg/L],
V_s—total volume of solution introduced into volumetric flasks [L],
V_{FPE}—volume of FPE introduced into volumetric flasks [L].

We used the same methods to evaluate the clove bud extract for comparison. The extraction of buds was carried out using the ultrasonic method. For this purpose, 20 g of ground plant material (in the amount of 6.40%) was introduced into 300 mL of distilled water, and extraction was carried out using an ultrasonic bath at 40 kHz (for 20 min at 40 °C), after which the extract obtained was filtered on a pressure funnel through a Whatman paper filter (EEA03).

2.11. Activated Carbons from Waste

Dried plant waste constituting clove buds was chemically activated with two chemicals (using KOH and H_2SO_4) and heat-treated. The adsorption properties of the activated carbon were studied in relation to the adsorption of methylene blue (MB) from an aqueous solution [37].

2.12. Statistical Analysis

During the optimisation of the fermentation process, a central composition plan (CCD) was used. The experimental plan and contour drawings were made using the computer program Statistica 13.3 PL software 7 (StatSoft, Kraków, Poland). A one-way analysis of variance (ANOVA) was used to statistically analyse the optimisation process. The adequacy of the function tested was checked using an ANOVA test. Results are displayed as the mean ± standard deviation (SD). Statistical computations were performed using Statistica 13.3 PL software 7 (StatSoft, Kraków, Poland).

Statistical analysis related to cytotoxicity tests of the results obtained was performed using GrahPad Prism 6.0 software (GraphPad Software, Inc., Sand Diego, CA, USA). The values of the parameters tested were expressed as the arithmetic mean ± standard deviation (SD). All results were subjected to a two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Statistical significance was determined by the following: **** $p < 0.0001$ *** $p < 0.001$ ** $p < 0.01$ * $p = 0.0320$ (the reduction in resazurin in keratinocytes HaCaT), **** $p < 0.0001$ *** $p = 0.0004$ ** $p = 0.0014$ (the reduction in resazurin in fibroblasts (HDF), *** $p = 0.0001$ ** $p < 0.01$ (the Neutral Red dye uptake in keratinocytes (HaCaT), ** $p = 0.0054$ * $p < 0.05$ (the Neutral Red dye uptake in fibroblasts (HDF) compared to the control.

3. Results

3.1. Studies of the Influence of Technological Parameters on the Fermentation of Clove Buds

Based on the results obtained by GC-MS, it was found that the highest lactic acid efficiency was obtained on 9 days in the case of FPE using *L. brevis* LY_1120 (LA_e = 72%) and FPE using *L. plantarum* MI-0102 (LA_e = 88%), and 10 days in the case of FPE using *L. reuteri* MI_0168 (LA_e = 94%), FPE using *L. salivarius* LY_0652 (LA_e = 65%), FPE using *L. acidophilus* MI-0078 (LA_e = 75%), and FPE using *L. rhamnosus* MI-0272 (LA_e = 96%). Therefore, *L. rhamnosus* strain MI-0272 was selected for further studies on the fermentation process of clove buds—Table S1.

Table S2 shows that the highest values of AA antioxidant activity (34.98 mmol Tx/L ± 0.03) can be achieved by running the fermentation process from 9 to 11 days, using an initial sugar content of 4.60%. As the initial sugar content is lowered (from 3.20 to 1.60%), there is a reduction in AA values from 33.90 mmol Tx/L ± 0.04 to 29.53 mmol Tx/L ± 0.03 from 9 to 11 days. This phenomenon is related to the fact that the starting beet molasses contains antioxidants (such as syringic acid, p-hydroxybenzoic acid, vanillic acid, ferulic acid, and others), which affect the increase in antioxidant activity in the fermented plant extract. The highest TPC values (11.68 mmol GA/L ± 0.09) can be achieved by running the fermentation process from 9 to 11 days, with an initial sugar content of 4.60%. Reducing the initial sugar content from 3.20 to 1.60% at lower process running times (from 1 to 5 days) results in lower TPC values from 10.74 mmol GA/L ± 0.03 to 8.78 mmol GA/L ± 0.03 (when running the fermentation for 1 day) and from 10.97 mmol GA/L ± 0.02 to 9.56 mmol GA/L ± 0.01 (when running the fermentation for 5 days). Table S2 shows that by using an initial sugar content of 3.20% for a process time of 9 to 11 days, it is possible to achieve the highest lactic acid yield of 95%. At the same time, increasing the initial sugar content regardless of the fermentation running time lowers the value of the studied function from 75 to 32%—Table S2.

Table S3 shows that the highest antioxidant activities are obtained using a plant material content of 6.40% and a fermentation time of 9 to 11days. Lowering the plant material content, regardless of the fermentation time, results in a decrease in the value of this function from about 1.73 mmol Tx/L ± 0.02 to about 19.94 mmol Tx/L ± 0.03. For a plant material content of 6.40% and a fermentation time of 9 to 11 days, it is possible to achieve the highest content of phenolic compounds in FPE. With the fermentation running time of 1 to 5 days (at the maximum plant raw material content), the value of this function gradually increases from 10.74 mmol GA/L ± 0.03 to 10.97 mmol GA/L ± 0.07. The use of lower vegetable raw material contents (from 0.32 to 3.20%) results in a decrease in TPC from 2.98 mmol GA/L ± 0.03 to 8.62 mmol GA/L ± 0.03. Table S3 also shows that increasing the plant material content at the selected fermentation time (from 9 to 11 days) results in a significant increase in TPC from about 3.76 mmol GA/L ± 0.04 (plant material content 0.32%) to about 8.62 mmol GA/L ± 0.03 (plant material content 3.20%). Table S3 shows that during 9 to 11 days of conducting the fermentation process, regardless of the amount of plant material used, it is possible to achieve the highest lactic acid efficiency. When conducting the fermentation of clove buds for 9 days using a plant material content of 1.60%, it is possible to achieve the highest value of this function (97%). Increasing the duration of the process with a constant content of plant matter results in a reduction in LA_e from 97 to 92%. For higher plant material contents of 3.20 and 6.40%, such a significant reduction in the value of this function is no longer observed—Table S3.

Table 2 shows the most favourable parameters for the fermentation process of clove buds in the presence of LAB and with beet molasses as a source of 6-carbon sugars.

3.2. Response Surface Method for Fermentation Process Optimisation

3.2.1. Screening of Fermentative Strains

The efficiency of lactic acid depending on the type of microorganism is shown in Figure 1.

Based on the results obtained by GC-MS, it was found that the highest lactic acid efficiency was obtained on 9 days in the case of FPE-3 (LA_e = 72%) and FPE-6 (LA_e = 88%), and 10 days in the case of FPE-1 (LA_e = 94%), FPE-2 (LA_e = 65%), FPE-4 (LA_e = 75%), and FPE-5 (LA_e = 96%). Therefore, L. rhamnosus strain MI-0272 was selected for further studies on the fermentation process of clove buds—Figure 1.

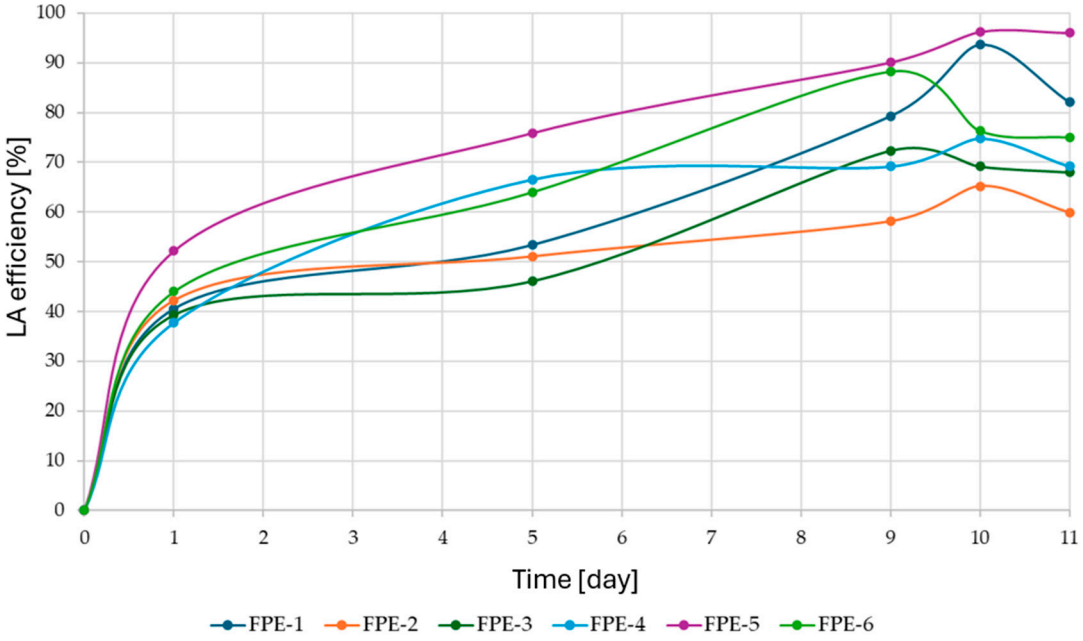

Figure 1. Lactic acid efficiency of fermented plant extracts: FPE-1, FPE-2, FPE-3, FPE-4, FPE-5, and FPE-6 obtained using 6 individual strains of lactic acid bacteria: 1. *L. reuteri* MI_0168, 2. *L. salivarius* LY_0652, 3. *L. brevis* LY_1120, 4. *L. acidophilus* MI-0078, 5. *L. rhamnosus* MI-0272, and 6. *L. plantarum* MI-0102. Process conducts parameters: initial sugar content 3.20%; inoculum content 3.20%; plant material content 1.6%.

3.2.2. Effect of Plant Raw Material Content and Fermentation Time

Figure 2 show the changes in antioxidant activity (AA), total polyphenols content (TPC), and lactic acid efficiency (LA_e) during the interaction of the studied process parameters.

Figure 2A, showing the relationship between plant material content and fermentation time, shows that the highest antioxidant activities are obtained using a plant material content of 6.40% and a fermentation time of 9 to 10 days. Lowering the plant material content, regardless of the fermentation time, results in a decrease in the value of this function from about 1.70 mmol Tx/L to about 19.90 mmol Tx/L (Figure 2A).

Figure 2B, showing the relationship between plant material content and fermentation time, shows that for a plant material content of 6.40% and a fermentation time of 9 to 10 days, it is possible to achieve the highest content of phenolic compounds in FPE. With the fermentation running time of 1 to 5 days (at the maximum plant raw material content), the value of this function gradually increases from about 10.70 mmol GA/L to about 10.90 mmol GA/L. The use of lower vegetable raw material contents (from 0.32 to 3.20%) results in a decrease in TPC from about 2.90 mmol GA/L to about 8.60 mmol GA/L. Figure 2B also shows that increasing the plant material content at the selected fermentation time (from 9 to 10 days) results in a significant increase in TPC from about 3.70 mmol GA/L (plant material content 0.32%) to about 8.60 mmol GA/L (plant material content 3.20%).

Figure 2C, showing the relationship between the content of plant material and the duration of the fermentation process, shows that during 9 to 11 days of conducting the fermentation process, regardless of the amount of plant material used, it is possible to achieve the highest lactic acid efficiency. When conducting the fermentation of clove buds for 9 days using a plant material content of 1.60%, it is possible to achieve the highest value of this function. Increasing the duration of the process with a constant content of plant material results in a reduction in LA_e from about 97 to about 92%. For higher plant material contents of 3.20 and 6.40%, such a significant reduction in the value of this function is no longer observed (Figure 2C).

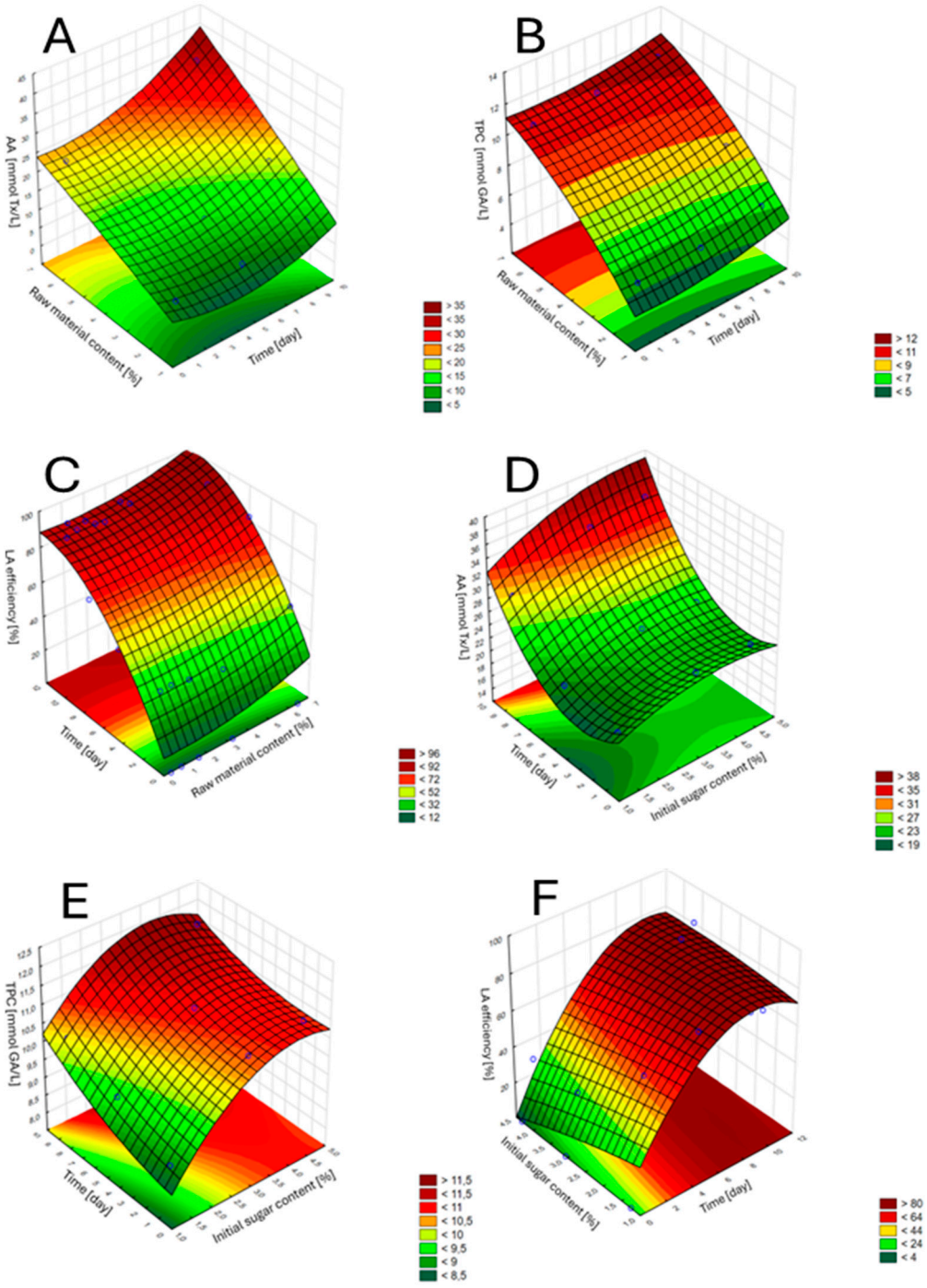

Figure 2. (**A**) changes in antioxidant activity (AA); (**B**) changes in total polyphenols content (TPC); (**C**) changes in lactic acid efficiency (LA_e), relationship: raw material content—reaction time. Constant parameters of the process: *L. rhamnosus* strain MI-0272; initial sugar content: 3.20%; inoculum content: 3.20%; (**D**) changes in antioxidant activity (AA); (**E**) changes in total polyphenols content (TPC); (**F**) Changes in lactic acid efficiency (LA_e), relationship: initial sugar content—reaction time. Constant parameters of the process: *L. rhamnosus* strain MI-0272; raw material content 6.40%; inoculum content: 3.20%.

Figure 2D, showing the relationship between changes in initial sugar content and fermentation time, shows that the highest values of AA antioxidant activity (about 34.90 mmol Tx/L) can be achieved by running the fermentation process from 9 to 10 days, using an initial sugar content of 4.60%. As the initial sugar content is lowered (from 3.20 to 1.60%), there is a reduction in AA values from about 33.90 mmol Tx/L to about 29.50 mmol Tx/L from 9 to 10 days. This phenomenon is related to the fact that the starting beet molasses contains antioxidants, which affect the increase in antioxidant activity in the fermented plant extract (Figure 2D).

Figure 2E, showing the relationship between changes in total polyphenol content and fermentation running time, shows that the highest TPC values (about 11.60 mmol GA/L) can be achieved by running the fermentation process for 9 to 10 days with an initial sugar content of 4.60%. Reducing the initial sugar content from 3.20 to 1.60% at lower process running times (from 1 to 5 days) results in lower TPC values from about 10.70 mmol GA/L to about 8.70 mmol GA/L (when running the fermentation for 1 day) and from about 10.90 mmol GA/L to about 9.50 mmol GA/L (when running the fermentation for 5 days)—Figure 2E.

Figure 2F, which shows the relationship between the initial sugar content and fermentation time, shows that by using an initial sugar content of 3.20% for a process time of 9 to 10 days, it is possible to achieve the highest lactic acid yield of 95%. At the same time, increasing the initial sugar content regardless of the fermentation running time lowers the value of the studied function from 75 to 32% (Figure 2F).

Table 3 shows the optimal parameters determined for the fermentation process of clove buds in the presence of LAB and beet molasses and the corresponding values of the main process functions.

Table 3. Optimal parameters for the fermentation process of clove buds in the presence of LAB and beet molasses and the corresponding values of the main functions of the process.

Optimal Parameters of the Fermentation Process	Unit	
Type of microorganism	-	*L. rhamnosus* MI-0272
Initial sugar content	%	3.20
Raw material content	%	6.40
Initial sugar content	days	9
Functions of the fermentation process		
AA	mmol Tx/L	33.90
TPC	mmol GA/L	11.60
LA_e	%	96

The results obtained from the fermentation of clove buds show that by conducting fermentation in the presence of *L. rhamnosus* MI-0272, it is possible to obtain a high efficiency of LA (96%). Using an initial sugar content of 3.20% with a plant raw material content of 6.40%, fermentation of 6-carbon sugars derived from beet molasses by LAB is possible. The results further show that the time of 9 days is conducive to the fermentation process, resulting in high LA efficiency with high AA and TPC values at the same time (Table 3).

Table 4 shows the most relevant response surface methodology (RSM) optimisation statistics.

Based on the determined statistical coefficients (Table 4), it can be concluded that for AA and TPC, the response surface described by the 2nd degree equation correlates very well with the experimental data. In the case of LA_e, fitting the response surface with the 2nd degree equation yields a weaker fit. Although in this case the response surface does not correlate well over the entire study area, its fit allowed us to determine the optimal areas of the fermentation process of *Syzygium aromaticum* L. *Myrtaceae* (Table 4).

Table 4. The most relevant response surface methodology (RSM) optimisation statistics.

	Effect of Plant Raw Material Content and Fermentation Time		
	AA	TPC	LA_e
a_0	3.59	2.49	10.37
a_1	−1.63	−0.05	−0.41
a_2	0.18	0.02	0.35
a_3	3.32	1.92	13.02
a_4	−0.06	−0.10	−0.54
a_5	0.19	0.00	−0.03
R^2	0.99	1.00	0.87
$AdjR^2$	0.97	1.00	0.84
SD	0.80	0.042	14
RSD	0.055	0.0044	0.42
PRESS	5.7	0.016	2980
	Effect of Initial Sugar Concentration and Fermentation Time		
	AA	TPC	LA_e
a_0	17.77	6.68	32.00
a_1	3.56	1.98	12.00
a_2	−0.49	−0.23	−0.71
a_3	−1.72	0.08	−5.00
a_4	0.27	0.01	−0.47
a_5	0.11	−0.02	0.74
R^2	1.00	0.98	0.80
$adjR^2$	0.99	0.95	0.69
SD	0.30	0.12	12
RSD	0.010	0.0099	0.35
PRESS	0.80	0.13	4488

The reliability of the response surface methodology for fermented plant extract obtained from *Syzygium aromaticum* L. *Myrtaceae* was confirmed experimentally. The obtained values for the main functions of the buds fermentation process according to the RSM analysis are shown in Table 5.

Table 5. The values for the main functions of the buds fermentation process according to the RSM analysis.

Functions of the Fermentation Process *	**Unit**	**Value**
AA	mmol Tx/L	33.89 ± 0.04
TPC	mmol GA/L	11.61 ± 0.02
LA_e	%	96 ± 1

* Constant parameters of the process: *L. rhamnosus* strain MI-0272; initial sugar content 3.20%; raw material content 6.40%; inoculum content 3.20%, and fermentation time of 9 days.

The reliability of the response surface methodology for the fermented plant extract obtained from *Syzygium aromaticum* L. *Myrtaceae* was confirmed experimentally, with the following values of the main process functions obtained: AA = 33.89 mmol Tx/L ± 0.04, TPC = 11.61 mmol GA/L ± 0.02, and LA_e = 96% ± 1 (Table 5).

3.3. Activity, Total Polyphenols Content, and LA Efficiency

Table 6 shows the results of the activity, total polyphenols content, and LA efficiency of optimised FPE and PE from clove buds.

Table 6. Presents the results for the activity, TPC, and LA efficiency of optimised FPE and PE from clove buds.

Cosmetic Raw Material	Chelating Activity ChA	Reduction Activity RA	Total Polyphenol Content TPC	Antioxidant Activity AA	LA Efficiency LA_e
	mmol Fe^{2+}/L FPE	mmol Fe^{3+}/L FPE	mmol GA/L	mmol Tx/L	%
FPE *	0.32 ± 0.01	49.09 ± 0.16	33.90 ± 0.14	11.60 ± 0.09	96
PE **	0.13 ± 0.01	35.42 ± 0.22	22.04 ± 0.13	9.21 ± 0.05	0

Mean ± SD (n = 6), * optimal parameters for the fermentation process of clove buds in the presence of LAB and beet molasses: type of microorganism (*L. rhamnosus* MI-0272), initial sugar content (3.20%), plant material content (6.40%), process time (10 days), ** parameters for the extraction process of clove buds using a 40 kHz ultrasonic bath: plant material content (6.40%), process time (20 min), temperature of extraction (40 °C).

Table 6 shows the antioxidant activity (via a DPPH radical scavenging assay), total polyphenol content (via the Folin–Ciocalteu method), Fe^{2+} chelating activity, Fe^{3+} reductivity, and LA efficiency of optimised FPE and PE. Antioxidant activity (AA) tests showed that the fermented plant extract had a higher degree of DPPH radical scavenging (11.60 mmol Tx/L ± 0.09) than the plant extract (9.21 mmol Tx/L ± 0.05). The results, presented in Table 6, show that the optimised FPE also has a higher content of phenolic compounds (TPC = 33.90 mmol GA/L ± 0.14) than the test extract (22.04 mmol GA/L ± 0.13). Studies of the chelating activity of Fe^{2+} ions and the reduction in Fe^{3+} ions in the ferrozine test showed that the activity of the tested FPE chelating and reduction potential is 0.32 mmol Fe^{2+}/L ± 0.01 and 49.09 mmol Fe^{3+}/L ± 0.16, while in the case of the PE, the ChA and RA values are lower (0.13 mmol Fe^{2+}/L ± 0.01 and 35.42 mmol Fe^{3+}/L ± 0.22). Based on the results obtained by GC-MS, it was found that the lactic acid efficiency was obtained on the 10th day of conducting the fermentation of clove buds: LA_e = 96% (Table 6).

3.4. Cytotoxicity Assessment

Figure 3 shows the effect of non-fermented and fermented extracts from *S. aromaticum* L. *Myrtaceae* on the reduction in resazurin in keratinocytes.

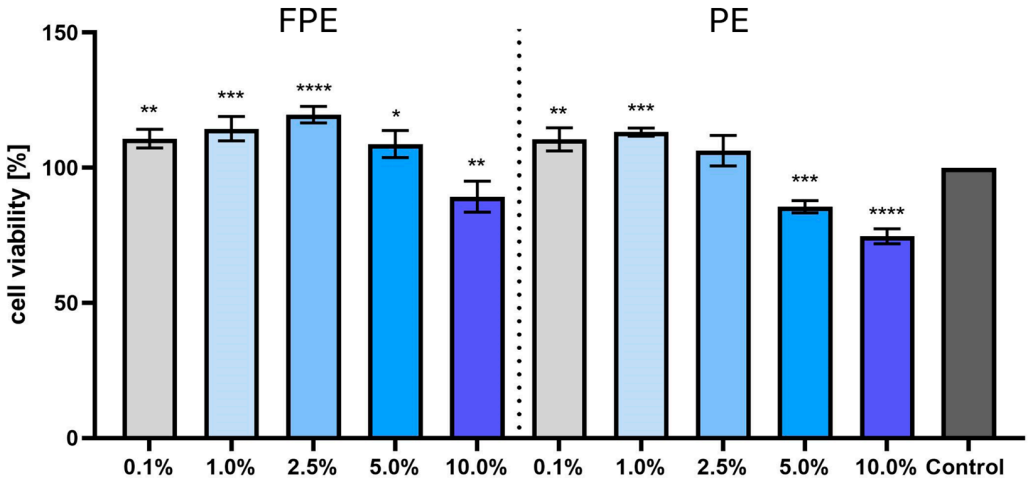

Figure 3. The effect of *S. aromaticum* L. *Myrtaceae* FPE and PE in concentrations of 0.1% (corresponding to 0.055 and 0.048 mg/mL); 1.0% (corresponding to 0.55 and 0.48 mg/mL); 2.5% (corresponding to 1.375 and 1.196 mg/mL); 5.0% (corresponding to 2.75 and 2.39 mg/mL); and 10.0% (corresponding to 5.5 and 4.8 mg/mL) on the reduction in resazurin in keratinocytes (HaCaT) after 24-h exposure. Control cells were keratinocytes untreated with the tested samples, for which viability was assumed to be 100%. Data are the means ± SD of three independent experiments, each consisting of three replicates per test group: **** $p < 0.0001$ *** $p < 0.001$ ** $p < 0.01$ * $p = 0.0320$.

Figure 4 shows the effect of extracts from *S. aromaticum* L. *Myrtaceae* on the reduction in resazurin in fibroblasts (HDF) after 24-h exposure.

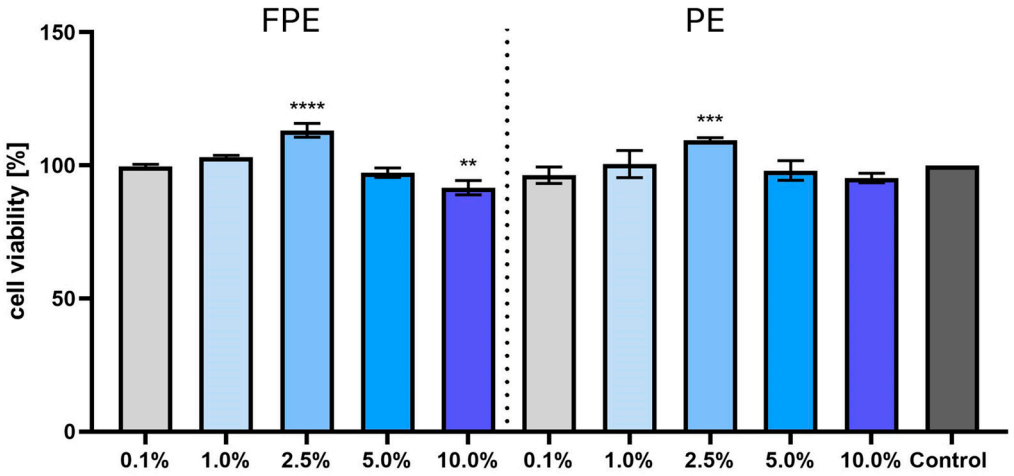

Figure 4. The effect of *S. aromaticum* L. *Myrtaceae* FPE and PE in concentrations of 0.1% (corresponding to 0.055 and 0.048 mg/mL); 1.0% (corresponding to 0.55 and 0.48 mg/mL); 2.5% (corresponding to 1.375 and 1.196 mg/mL); 5.0% (corresponding to 2.75 and 2.39 mg/mL); and 10.0% (corresponding to 5.5 and 4.8 mg/mL) on the reduction in resazurin in fibroblasts (HDF) after 24-h exposure. Control cells were fibroblasts untreated with the tested sample, for which viability was assumed to be 100%. Data are the means ± SD of three independent experiments; each consisting of three replicates per test group: **** $p < 0.0001$ *** $p = 0.0004$ ** $p = 0.0014$.

The cytotoxicity of non-fermented and fermented extracts from *S. aromaticum* L. *Myrtaceae* was also assessed using the Neutral Red assay, which assessed the ability of cells exposed to PE and FPE to bind a cationic dye (neutral red) in the lysosomes of the tested cells [26].

Figure 5 shows the effect of extracts from *S. aromaticum* L. *Myrtaceae* on the Neutral Red dye uptake in keratinocytes (HaCaT) after 24-h exposure.

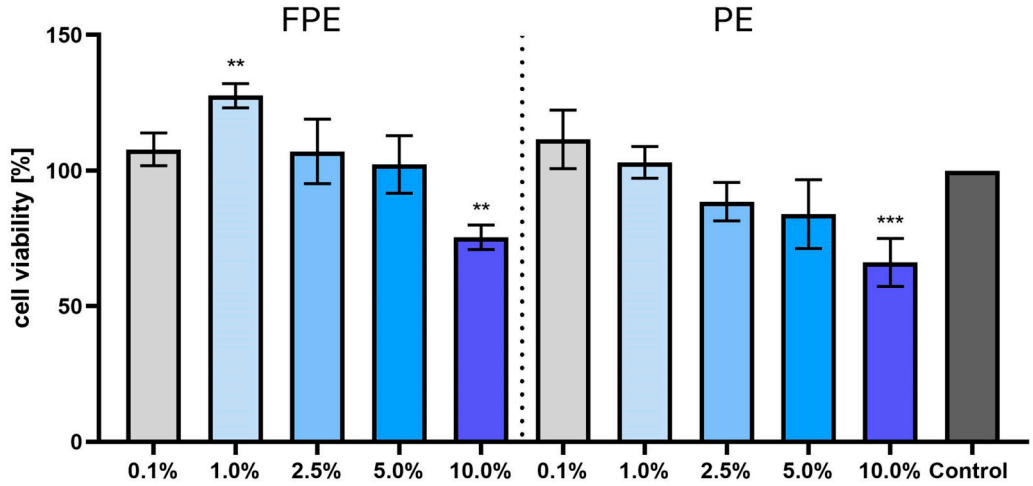

Figure 5. The effect of *S. aromaticum* L. *Myrtaceae* FPE and PE in concentrations of 0.1% (corresponding to 0.055 and 0.048 mg/mL); 1.0% (corresponding to 0.55 and 0.48 mg/mL); 2.5% (corresponding to 1.375 and 1.196 mg/mL); 5.0% (corresponding to 2.75 and 2.39 mg/mL); and 10.0% (corresponding to 5.5 and 4.8 mg/mL) on the Neutral Red dye uptake in keratinocytes (HaCaT) after 24-h exposure. Control cells were keratinocytes untreated with the tested samples, for which viability was assumed to be 100%. Data are the means ± SD of three independent experiments; each consisting of three replicates per test group: *** $p = 0.0001$ ** $p < 0.01$.

Figure 6 shows the effect of extracts from *S. aromaticum* L. *Myrtaceae* on the Neutral Red dye uptake in fibroblasts (HDF) after 24-h exposure.

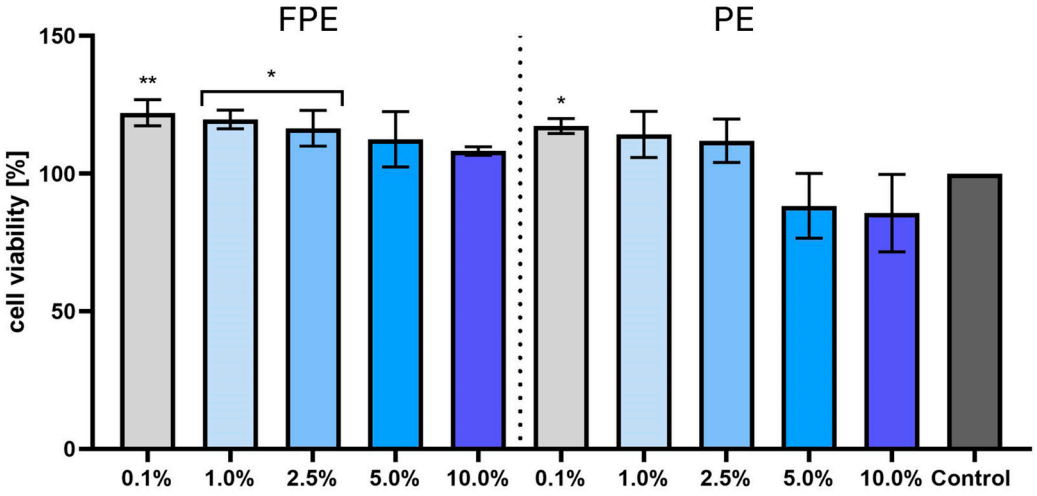

Figure 6. The effect of *S. aromaticum* L. *Myrtaceae* FPE and PE in concentrations of 0.1% (corresponding to 0.055 and 0.048 mg/mL); 1.0% (corresponding to 0.55 and 0.48 mg/mL); 2.5% (corresponding to 1.375 and 1.196 mg/mL); 5.0% (corresponding to 2.75 and 2.39 mg/mL); and 10.0% (corresponding to 5.5 and 4.8 mg/mL) on the Neutral Red dye uptake in fibroblasts (HDF) after 24-h exposure. Control cells were keratinocytes untreated with the tested samples, for which viability was assumed to be 100%. Data are the means ± SD of three independent experiments; each consisting of three replicates per test group: ** $p = 0.0054$ * $p < 0.05$.

Both PE and FPE might boost skin cell viability in vitro. HaCaT cells showed the largest pro-proliferative effect at a concentration of 2.5% (1.375 mg/mL) for FPE, with a vitality of 119.6%, and the most ideal concentration for PE was 1.0% (0.48 mg/mL), with a viability of 114.4%. In the case of HDF cells, the best concentration for both FPE and PE was 2.5% (corresponding to 1.375 and 1.196 mg/mL), with viability of 113.2% and 109.5%. Keratinocytes reported a maximum viability of 127.5% at a concentration of 1.0% (0.55 mg/mL) after using FPE. In the case of PE, we recorded the maximum cell activity (111.5%) at a concentration of 0.1% (0.048 mg/mL), but as the concentration increased, the viability gradually decreased, reaching 66.1% at a concentration of 10.0% (4.8 mg/mL). In the case of fibroblasts, all of the tested FPE concentrations of 0.1–10.0% (0.055–5.5 mg/mL) increased viability when compared to control cells. At a concentration of 0.1%, we reported the highest viability (122.1%); however, as the concentration of FPE increased, the viability gradually declined, reaching 108.2% at a concentration of 10.0%. In the case of PE, doses up to 2.5% (up to 1.196 mg/mL) had a favourable effect on viability. Comparing the results of both experiments, it is possible to conclude that FPEs have a greater favourable influence on the vitality and activity of the examined cells, as evidenced by a stronger pro-proliferative effect and lower cytotoxicity (Figures 3–6).

3.5. Obtaining Activated Carbons from Waste

Clove buds constituting the fermentation waste were carbonised at 600 °C for 20 min (sample A-0). For this purpose, 100 g of dried plant waste was placed in a steel reactor equipped with a system for receiving the gaseous products of pyrolysis. A GOLD-BRUNN crucible furnace and a cooler were used. The obtained carbonizates were subjected to activation according to the procedure described in another publication [38].

Sample A-1 was activated as follows: 10 g of grated carbonizate was soaked for 24 h in 20 mL of a 50% solution of sulfuric acid VI. Then the sample was centrifuged and decanted. The carbonizate thus modified was placed in a steel reactor and heated in a crucible furnace to 800 °C. After reaching the desired temperature, the carbonizate was kept at this temperature for 10 min. After this time, the sample was cooled to room temperature and washed with distilled water until the pH was neutral.

Sample A-2 was activated as follows: to 10 g of grated carbonate was added 5 g of KOH. The whole thing was grated. The carbonizate thus modified was placed in a steel reactor and heated in a crucible furnace to 800 °C. After reaching the desired temperature, the carbonizate was kept at this temperature for 10 min. After this time, the sample was cooled to room temperature and washed with distilled water until the pH reached neutral.

In the case of sample A-3, 10 mL of 50% KOH solution was added to 10 g of grated carbonizate, and the whole thing was grated. The carbonizate thus modified was placed in a steel reactor and heated in a crucible furnace to 800 °C; after reaching the desired temperature, the carbonizate was kept at this temperature for 10 min. After this time, the sample was cooled to room temperature and washed with distilled water until the pH was neutral.

Next, the adsorption properties of activated carbonizates (A-1, A-2, and A-3) were investigated using methylene blue. MB blue adsorption studies were carried out using a static method. For this purpose, 200 mg of the corresponding carbonizate was introduced into conical flasks containing 500 mL of distilled water, and then 5 mL of aqueous methylene blue solution (with a concentration of C = 1000 mg/L) was added in portions, until a permanent coloration of the solution appeared. The system was stirred on a magnetic stirrer for 24 h. Using the mass balance Equation (8), we determined the amount of adsorbed methylene blue after this time:

$$a = \frac{\left(\frac{n \cdot V_{MB} \cdot C_{MB}}{n \cdot V_{MB} + V_W} - C_e\right) \cdot (n \cdot V_{MB} + V_W)}{m} \quad (8)$$

where

C_{MB}—concentration of methylene blue introduced into conical flasks (1000 mg/L),
C_e—final concentration of methylene blue introduced into conical flasks (mg/L),
m—adsorbent mass (0.2 g),
n—the number of portions of methylene blue introduced into conical flasks.
V_{MB}—volume of methylene blue each time introduced into conical flasks (5 mL),
V_W—water volume (500 mL).

The final MB concentration was determined photometrically with a Thermo Scientific Genesys 50 spectrophotometer at 664 nm using a glass cuvette with an optical path of 1 cm.

Figure 7 shows the adsorption magnitudes of methylene blue for the initial carbonizate (sample A-0) and for the activated carbonates (samples A-1–A-3).

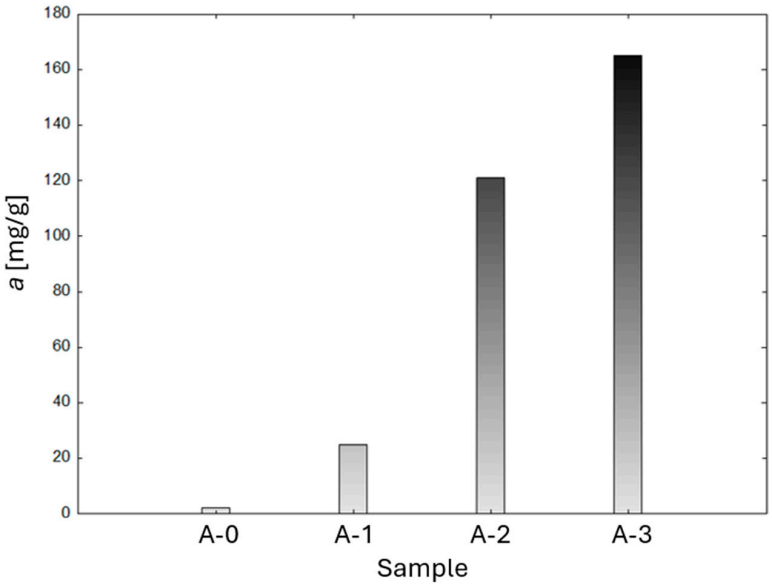

Figure 7. The magnitude of adsorption of methylene blue for the initial carbonizate (sample A-0) and for the activated (samples A-1, A-2, and A-3).

The initial carbonizate (sample A-0) shows an adsorption capacity of 2.3 mg/g. Modification of the carbonizate with sulfuric acid and potassium hydroxide increased the adsorption capacity of the obtained adsorbents (A-1, A-2, and A-3)—Figure 7.

4. Discussion

By running the process under the most favourable conditions, it is possible to obtain lactic acid (LA) as the main product with the highest efficiency of 96%, and further running the process affects the gradual decrease in LA in fermented plant extracts. The study of the influence of technological parameters on the fermentation process of clove buds shows that favourable results are obtained by conducting fermentation in the presence of *L. rhamnosus* strain MI-0272, using an initial sugar content of 3.20% (in terms of the amount of molasses of 40.32 g), a plant material content of 6.40% (64.50 g), and a time of 10 days (Tables S1–S3). The fermentation process of buds produces a fermented plant extract (FPE) with the highest antioxidant activity (AA) of 33.90 mmol Tx/L ± 0.04 and the highest content of phenolic compounds (TPC) of 11.60 mmol GA/L ± 0.04. Lactic acid bacteria are microorganisms of industrial importance that are known for their fermentation abilities, mainly due to their probiotic benefits as well as their production of lactic acid. When fermentation is carried out, LA production is inhibited, and a gradual decrease in the growth rate of lactic acid bacteria cells is observed [39]. Consequently, a gradual decrease in lactic acid concentration is observed as the process is prolonged. Lactic acid inhibition is caused by the ability of undissociated lactic acid to dissolve in the cytoplasmic membrane, while dissociated lactate remains insoluble. This leads to acidification of the cytoplasm and disruption of proton motive forces. This event affects the pH gradient across the cell membrane and reduces the cellular energy available for growth (Tables 1 and 2). Polyphenolic glucosides contained in clove buds are converted into more bioactive compounds (anthocyanin glucosides to their aglycone forms and free phenolic acids), thus leading to a higher content of phenolic compounds in fermented plant extracts. In addition, the simpler structure of the derived metabolites contributes to the higher antioxidant activity of FPEs, with an increase in their ability to reduce DPPH radicals relative to non-fermented extracts [40–42].

Optimisation of technological parameters on the fermentation of clove buds in the presence of *L. rhamnosus* MI-0272 showed that the highest values of the tested functions (AA, TPC, and LA_e) were obtained on day 9 of the process, using a plant material content of 6.40% and an initial sugar content of 3.20%. This yields a fermented plant extract characterised by high antioxidant activity (AA) and a high content of polyphenolic compounds (TPC). Increasing the content of the plant material (above 3.20%) hinders the hydrolysis of the 6-carbon sugars, with the result that incomplete hydrolysis of the sugars is observed [43]. This significantly reduces the efficiency of LA formation during fermentation. Using an optimal initial sugar concentration of 3.20%, lactic acid is obtained with a maximum efficiency of 96% (Tables 3 and 5; Figures 1 and 2). Lactic acid efficiency values first increased during the process, then reached a maximum on day 9 or 10 of fermentation (depending on the strain used). Continuing the fermentation process resulted in a decrease in the efficiency of the lactic acid formed. When conducting fermentation, inhibiting LA production often occurs, and a gradual decrease in the growth rate of lactic acid bacteria cells is observed. Therefore, a gradual decrease in lactic acid concentration is observed as the process continues. The inhibition of lactic acid is caused by the ability of the undissociated lactic acid to dissolve in the cytoplasmic membrane, while the dissociated lactate remains insoluble. This leads to acidification of the cytoplasm and the disruption of proton motive forces. This event impacts the pH gradient across the cell membrane and reduces the cellular energy available for growth [42]. Comparing the influences of the type of strain used on the efficiency of the LA obtained, it was found that the use of *L. rhamnosus* MI-0272 as the fermentation strain allowed lactic acid to be obtained with the highest efficiency (96%). Therefore, *L. rhamnosus* MI-0272 was selected as the superior fermentation strain. The results showed that the ferment obtained using *Rhodotorula glutinis* and *L. casei* had the highest lactic acid

content (15 g/L) [2]. Our study showed that all fermented plant extracts obtained had a higher lactic acid content (about 22 g/L).

Fermentation of black soybeans in the presence of *Bacillus subtilis* BCRC 14715 and using such solvents as water, methanol, ethanol, and acetone increased the total phenolic and flavonoid content, as well as the antioxidant activity of the obtained extracts. Phenols of different classes are converted into compounds that are more bioactive than the parent compounds, leading to higher contents of TPC and AA [40]. The fermented acetone extract had the highest total phenolic content of 40.42 ± 0.48 mg GA/g and the highest DPPH free radical scavenging effect (EC_{50} = 0.65 ± 0.02 mg/mL), while the fermented methanol extract showed the highest Fe^{2+} ion chelating capacity (EC_{50} = 2.17 ± 0.19 mg/mL). The active substance associated with the DPPH radical scavenging effect was most efficiently extracted from black soybeans using water as a solvent. Water effectively extracted iron (II) ion chelating principles from unfermented (yield = 44.20% ± 0.21) and fermented (yield = 50.79% ± 1.98) black soybeans, respectively [44]. Fermentation of cheonggukjang soybeans by *Bacillus subtilis* CS90 probiotics increased total polyphenol content (from 253 to 9414 mg/kg) and DPPH radical scavenging activity (from 53.6 to 93.9%), while total flavanol gallate content decreased by about 70% over the 60-h fermentation period. As fermentation time increased, significant differences in total phenolic acids were observed: 311.01 mg/kg (time = 0 h), 400.45 mg/kg (time = 12 h), 508.48 mg/kg (time = 24 h), 733.59 mg/kg (time = 36 h), 890.05 mg/kg (time = 48 h), 1066.92 mg/kg (time = 60 h). In particular, the level of phenolic acids increased significantly with a decrease in flavanol gallates during the fermentation run. Total phenolic content (TPC) is higher after fermentation of plant raw materials in the presence of LAB microorganisms, which can be explained by the generation of metabolites and the release of phenols from the plant matrix. However, in some cases, TPC reduction after fermentation is more common due to enzymatic or non-enzymatic oxidation, diffusion of soluble phenolic compounds into fermentation secretions (exudates), or condensation reactions with proteins. In addition, the total phenolic content may increase or decrease depending on the strains of microorganisms [8,41,45].

During the ongoing fermentation of plant raw materials, it has been observed that gallates are converted into flavanols (epigallocatechin gallate into epigallocatechin). In addition, flavanols (catechin, epicatechin, and epigallocatechin) exhibit antiviral, antimutagenic, anticancer, anti-obesity, and hypocholesterolemic effects. Flavanols are also considered effective antioxidants, and they work by scavenging oxygen radicals and chelating metal ions (for example, Fe^{2+} ions). Phenolic acids, such as gallic acid and its derivatives, also have antioxidant, antimutagenic, and anticancer effects. Gallic and rosemary acids were the most potent antioxidants among simple phenolic and hydroxycinnamic acids. A review of the literature indicates that phenolic acid content correlates with the antioxidant capacity of FPEs [45,46].

Degradation of anthocyanin glucosides to their aglycone forms and free phenolic acids is also observed during fermentation in the presence of lactic acid bacteria. Fermentation of jussar pulp by *L. deubruekii*, assisted by the action of β-glucosidase, led to the conversion of cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside to the main product, protocatechuic acid, which is a phenolic compound. In addition, other phenolic acids, such as caffeic acid and ferulic acid, were detected in smaller amounts within a few hours of fermentation, indicating possible conversion between phenolic acids via chalcones. The simpler structure of these metabolites may contribute to the higher antioxidant activity of fermented jussar pulp, with an increase in oxygen radical-reducing capacity from 49.2 to 90.7% compared to the unfermented extract. Accordingly, *L. plantarum* and *Oenococcus oeni* deglycosylate anthocyanin glucosides into their aglycones: malvidin, delphinidin, and peonidin. Further ring cleavage released trihydroxybenzaldehyde and syringic acid (from malvidin), gallic acid (from delphinidin or demethylation of syringic acid), or vanillic acid (from peonidin) [40,47].

The degree of anthocyanin degradation by lactic acid bacteria depends on the LAB species. The highest anthocyanin content was observed by conducting fermentation in

the presence of *L. casei* and *L. bulgaricus*, while conducting fermentation in the presence of other *L. species* resulted in anthocyanins at lower levels. In addition, the anthocyanin content, particularly delphinidin 3-O-glucoside, in the fermented orange juice was more significantly reduced by *L. rhamnosus* or *L. paracasei* than by *L. plantarum* or *L. brevis*. These observations may be due to variability in β-glucosidase or β-galactosidase activity, and the presence of more hydroxyl groups in delphinidin increases its susceptibility to enzymatic degradation more than that of other anthocyanins [39,48].

The cytotoxicity of plant extracts is closely dependent on the part of the plant being extracted and the phytochemicals contained in that part of the plant material [49,50]. When assessing the potential use of individual plant raw materials in preparations applied to the skin, it is extremely important to assess their cytotoxicity in vitro, which allows for a preliminary assessment of the validity and possibility of using the analysed plant as a cosmetic ingredient [51]. In order to assess the impact of the obtained PE and FPE from *S. aromaticum* L. *Myrtaceae* on the viability and metabolism of skin cells, an analysis of their cytotoxicity on keratinocytes (HaCaT) and fibroblasts (HDF) was performed. As part of the analyses, the Alamar blue test was carried out, which measures the level of reduction in the non-fluorescent blue resazurin dye to the red fluorescent dye, which allows for the assessment of the level of metabolic activity of cells [25]. The results obtained in the resazurin reduction test indicated a concentration-dependent effect of the tested samples on the viability of keratinocytes and fibroblasts. This test indicated that both PE and FPE could increase skin cell viability in vitro. The strongest pro-proliferative effect in HaCaT cells was recorded at a concentration of 1.375 mg/mL (in the case of FPE), at which the viability was 119.6%, while for the extract, the most optimal concentration was 0.48 mg/mL, which increased the viability of these cells to 114.4%. In the case of HDF cells, the most optimal concentrations for FPE and PE were 1.375 and 1.196 mg/mL, respectively, for which the viability was 113.2% and 109.5%. However, note that increasing the concentration of both FPE and PE to 2.75–5.5 mg/mL and 2.39–4.8 mg/mL results in a decrease in the viability of the tested cells, indicating the cytotoxic effect of these samples at higher concentrations. In the case of the Neutral Red assay, it was shown that the effect of the tested fermented and non-fermented extracts, similarly to the first test, varies depending on the concentration of PE used and the type of cells. For keratinocytes, the use of FPE resulted in the highest viability at a concentration of 0.55 mg/mL, achieving a cell viability of 127.5%. In the case of PE, the highest cell activity (111.5%) was recorded for a concentration of 0.048 mg/mL, while with the increase in concentration, the viability gradually decreased, reaching 66.1% for a concentration of 4.8 mg/mL. In the case of fibroblasts, all of the tested concentrations (0.055–5.5 mg/mL) of FPE resulted in an increase in viability compared to control cells. The highest viability (122.1%) was recorded for a concentration of 0.055 mg/mL, while with the increase in concentration, the viability gradually decreased, reaching a value of 108.2% at a concentration of 5.5 mg/mL. However, in the case of PE, concentrations up to 1.196 mg/mL had a positive effect on viability, while higher concentrations caused a cytotoxic effect. Comparing the results obtained in both tests, it can be concluded that FPEs have a more beneficial effect on the viability and activity of the tested cells, for which a stronger pro-proliferative effect and lower cytotoxicity were observed (Figures 3–6). The cytotoxicity activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against human skin fibroblast 1184 cells was used to determine the relative cell viability of the plant extract from *Syzygium aromaticum*. The highest percentage of relative cell viability for *S. aromaticum* extract was observed at a concentration of 10 ppm [52].

When considering the use of *S. aromaticum* L. *Myrtaceae* extracts in topical cosmetic preparations, appropriate concentrations should be used to eliminate their cytotoxic effects [53]. Prashar et al. [54] confirmed the cytotoxic effect of essential oil from *S. aromaticum* L. on dermal fibroblasts [54]. When clove oil was tested on HDF cell lines, it was found to be highly cytotoxic at concentrations as low as 0.03% (*v*/*v*) because it contained eugenol. On the other hand, β-caryophyllene, which is a part of CEO, did not show any cytotoxic

activity [54]. Also, eugenol concentrations that were cytotoxic led to less ATP and more glycolytic metabolites and polyamines in both normal oral cells and oral squamous cell carcinoma, which shows that unplanned cell death was triggered. Clove oil should be applied topically, at very low concentrations [53]. Studies of the effects of clove bud oil on skin fibroblasts showed that CEO had cytotoxicity at IC_{50} = 0.162 µL/mL [54].

In our study, no cytotoxicity was observed on fibroblasts treated with fermented bud extracts (Figures 3–6), suggesting that FPE from *S. aromaticum* L. *Myrtaceae* may improve metabolism and skin cell viability. Kim et al. [55] observed no cytotoxicity on fibroblasts treated with extracts from *S. aromaticum* L. in the concentration range of 0–200 µg/mL [55]. These results indicate that cytotoxicity is strictly dependent on both the part of the plant and the phytochemicals it contains [49,50]. So far, there are no literature reports on the cytotoxicity of ferments from *S. aromaticum* L. *Myrtaceae*, so the present work indicates for the first time their potential execution in the cosmetic and pharmaceutical industry.

The initial carbonizate (sample A-0) showed very poor adsorption capacity at 2.3 mg/g. Modification of the carbonizate with sulfuric acid (sample A-1) resulted in a more than 10-fold increase in adsorption capacity to a level of 25 mg/g. Modification of the carbonizate with potassium hydroxide (sample A-2) resulted in a more than 50-fold increase in adsorption capacity to a level of 121 mg/g, while modification of the carbonizate with the KOH impregnation method resulted in an increase in adsorption volume to 165 mg/g. The proposed activation methods effectively developed the specific surface area of the carbonizate and thus influenced the adsorption efficiency. Plant biomass waste subjected to appropriate modifications can be an attractive raw material for the production of adsorbents (Figure 7).

These studies are a prelude to further research on the use of FPE and PE from *S. aromaticum* L. *Myrtaceae* in cosmetic and pharmaceutical preparations. In addition to the optimisation performed, it is necessary to extend the analysis of the obtained FPE with regard to the content of individual phenolic acids in order to identify the compounds responsible for the therapeutic effect. Further studies are also necessary to determine the content of flavonoids reduced during fermentation and comprehend the mechanisms and metabolism of flavonoid degradation.

5. Conclusions

A detailed study of the influence of technological parameters using a single variable method made it possible to determine the technological parameters at which lactic acid contained in fermented plant extracts is formed with the highest efficiency (96%), with the highest values of antioxidant activity (33.90 mmol Tx/L ± 0.04) and total polyphenol content (11.60 mmol GA/L ± 0.04) at the same time.

By mathematically optimising the fermentation process of clove buds in the presence of LAB and beet molasses, we were able to determine the optimal parameters that yielded the highest values for the studied AA, TPC, and LA_e functions. The results show that by conducting fermentations over 9 days using a plant raw material of 6.40% and an initial sugar content of 3.20%, it is possible to obtain lactic acid with the highest efficiency while at the same time having high AA and TPC values. Moreover, the most favourable parameters for running the fermentation process were correlated with the optimal parameters.

The fermented plant extract obtained from *S. aromaticum* L. *Myrtaceae* is a valuable cosmetic raw material with high antioxidant potential (AA > 33 mmol Tx/L) that is safe for fibroblasts and keratinocytes (in concentrations of 5.0% corresponding to 2.75 mg/mL). Due to the presence of lactic acid (about 22 g/L), FPE can also be used in dermatological and cosmetic products applied to the skin with moisturising and regenerative effects.

The results presented in this article may encourage other scientists to undertake new research on the biotransformation of less studied classes of phenolic compounds and to develop optimal fermentation conditions for plant raw materials in order to maximise the efficiency of phenolic compounds in cosmetic raw materials, along with their high biological activity.

However, there are some limitations to this study. Although the resulting FPE has been optimised, detailed analyses related to the evaluation of the content of the individual phenolic acids responsible for the antioxidant activity of the FPE are still lacking. Furthermore, it is also important to study the kinetics of fermentation in order to select appropriate kinetic models that best describe the changes taking place during ongoing fermentation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14114763/s1, Table S1. Studies on the effect of the type of microorganism (*L. reuteri* MI_0168, *L. salivarius* LY_0652, *L. brevis* LY_1120, *L. acidophilus* MI-0078, *L. rhamnosus* MI-0272, and *L. plantarum* MI-0102) on changes in antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA_e); Table S2. Studies of the effect of the initial amount of sugars on changes in antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA_e); Table S3. Studies on the effect of plant raw material content changes: antioxidant activity (AA), total polyphenol content (TPC), and lactic acid efficiency (LA_e); Figure S1. A scatter plot of observed and approximated values during the interaction of the process parameters under study: raw material content—reaction time (changes in AA). Constant parameters of the process: *L. rhamnosus* strain MI-0272; initial sugar content: 3.20%; inoculum content: 3.20%; Figure S2. A scatter plot of observed and approximated values during the interaction of the process parameters under study: raw material content—reaction time (changes in TPC). Constant parameters of the process: *L. rhamnosus* strain MI-0272; initial sugar content: 3.20%; inoculum content: 3.20%; Figure S3. A scatter plot of observed and approximated values during the interaction of the process parameters under study: raw material content—reaction time (changes in LA_e). Constant parameters of the process: *L. rhamnosus* strain MI-0272; initial sugar content: 3.20%; inoculum content: 3.20%; Figure S4. A scatter plot of observed and approximated values during the interaction of the process parameters under study: initial sugar content—reaction time (changes in AA). Constant parameters of the process: *L. rhamnosus* strain MI-0272; raw material content 6.40%; inoculum content: 3.20%; Figure S5. A scatter plot of observed and approximated values during the interaction of the process parameters under study: initial sugar content—reaction time (changes in TPC). Constant parameters of the process: *L. rhamnosus* strain MI-0272; raw material content 6.40%; inoculum content: 3.20%; Figure S6. A scatter plot of observed and approximated values during the interaction of the process parameters under study: initial sugar content—reaction time (changes in LA_e). Constant parameters of the process: *L. rhamnosus* strain MI-0272; raw material content 6.40%; inoculum content: 3.20%.

Author Contributions: Conceptualisation, E.K.; methodology. E.K., M.J. and M.Z.-D.; software, R.P.; formal analysis, E.K., M.Z.-D. and R.P.; investigation, E.K., M.J., M.Z.-D. and R.P.; resources, E.K.; data curation, E.K., D.W. and P.M.; writing—original draft preparation, E.K.; writing—review and editing, E.K.; visualisation. E.K.; supervision, P.B. and S.K.; project administration, E.K.; funding acquisition, E.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Paweł Bilewicz, Sebastian Kowalczyk were employed by Dancoal Sp. z o.o. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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