

Microwave-Assisted Synthesis of Lactose Acetates with Antimicrobial, Cytotoxic, and Antiviral Properties

Radka Arabadzhieva¹ , Ivan Ivanov¹ , Nadezhda Petkova^{1*} , Mina Todorova²,
Yulian Tumbarski³ , Ivanka Vlaeva⁴ , Neli Vilhelmova-Ilieva⁵, Ivanka Nikolova⁵, Lora Simeonova⁵

¹ Department of Organic Chemistry and Inorganic Chemistry, University of Food Technologies, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria, radka_grudeva@outlook.com (R.A.); petkovanadejda@abv.bg (N.P.); ivanov_ivan.1979@yahoo.com (I.I.);

² Department of Organic Chemistry, Plovdiv University Paisii Hiledarski, 24 Tsar Asen Str., 4000, Plovdiv, Bulgaria; mm_todorova@abv.bg (M.T.);

³ Department of Microbiology, University of Food Technologies, 26 Maritza Blvd., 4002, Plovdiv, Bulgaria; tumbarski@abv.bg (Y.T.);

⁴ Department of Mathematics and Physics, University of Food Technologies, 26 Maritza Blvd., 4002, Plovdiv, Bulgaria; ivlaeva@yahoo.com (I.V.)

⁵ Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 26 G. Bonchev Str., 1113 Sofia, Bulgaria; nelivili@gmail.com (N.V-I.); vanianik@mail.bg (I.N.); losimeonova@gmail.com (L.S.)

* Correspondence: petkovanadejda@abv.bg (N.P.);

Scopus Author ID 56507003400

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Abstract: The task of this study was to perform the "green" synthesis of lactose octaacetate through microwave irradiation and to establish their biological activities. Lactose ester was prepared after microwave-assisted esterification of lactose with acetic anhydride (yield 85-90 %). Lactose octaacetate was characterized by a high degree of acetylation (DS 3.2-3.7). The lactose esters' structure was elucidated by infrared spectroscopy and nuclear magnetic resonance spectroscopy. Lactose octaacetate showed better antifungal activities than antibacterial activities. It possessed slight to moderate antifungal activities against *Aspergillus niger* ATCC 1015, *Penicillium* sp., *Rhizopus* sp., *Fusarium moniliforme* ATCC 38932. Lactose acetates demonstrated low cytotoxicity against three cell lines: Madin-Darby bovine kidney (MDBK) cells, human epithelial type 2 (HEp-2), and Madin-Darby canine kidney (MDCK) cells. This is the first report for antiviral activity of lactose acetates against herpes simplex virus type 1 (HSV-1), influenza virus A/Panama/2007/99/H3N2 - (IAV/H3N2), PV-1 and Coxsackievirus B1. It was found that this compound showed activity with SI = 2.4 only against PV-1, but against HSV-1, IAV/H3N2, and Coxsackievirus B1 was inactive. The current study demonstrated the applications of lactose acetates as antimicrobial and antiviral substances in food, pharmaceutical, agricultural and cosmetic preparations.

Keywords: lactose octaacetates; microwave irradiation; antimicrobial properties, antiviral activity

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1. Introduction

At the end of the 20th century, the 12 principles of Green Chemistry were postulated by Paul Anastas and John Warner. These principles impose new rules on the synthesis and analysis of chemicals. This fact marks the beginning of a new era in which industry and the environment are more linked than ever [1, 2].

For an extended time, the chemists believed that substances only reacted once they were in a liquid state or dissolved in an exceedingly solvent [3]. The foremost challenging problem

faced by the industry has been the employment of solvents. Recently, many efforts have been made to substitute or decrease the use of solvents by using microwaves, and materials that might absorb or adsorb reactants, as perform chemical reactions within microwave irradiation.

In modifying carbohydrates, ultrasonic [4-7] and microwave irradiation [8-11] were successfully used to accelerate the esterification process.

Chemical synthesis under microwave irradiation is an alternate method for preparing chemical compounds without the utilization of any solvents. Microwave irradiation has numerous advantages over the standard method: the speed of the process increases, respectively the reaction time decreases, the high purity final products, absence of solvent, heat loss in the environment can be avoided [12]. Microwaves have wavelengths between 0.01 and 1 meter and operate in a frequency range between 0.3 and 30 GHz. They are in the electromagnetic spectrum between infrared waves and radio waves [13]. Unlike conventional heating methods, microwave technology enables to achieve cleaner and more efficient chemical reactions with higher yields. Microwave irradiation in chemistry has become a popular technology exploited in medicinal chemistry, nanotechnology, and biochemical processes [14].

Sugar esters are a class of non-ionic, biocompatible, and biodegradable surfactants. They found application in pharmacy, foods, and cosmetic industries [15]. Lactose [β -D-galactopyranosyl-(1 \rightarrow 4)- α , β -D-glucopyranose] is a disaccharide, and the main sugar occurs in milk and whey its content reaches about 5 %. Whey is a waste product that causes serious environmental concerns. Therefore, the isolation of lactose as a by-product of the dairy industry and its further modification can be converted into added-value products [16]. Its peracetates (1,2,3,6,2',3',4',6'-octa-O-acetyl- α , β -lactose,2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-(1 \rightarrow 4)-1,2,3,6-tetra-Oacetyl- α , β -D-glucopyranose, β -lactose peracetate) is an important intermediate in synthetic carbohydrate chemistry [17]. Lactose octaacetate (peracetylated lactose), know also as 4-O-(2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl)-1,2,3,6-tetra-O-acetyl-D-glucopyranose; Octa-O-acetyl-D-lactose; 4-O-(2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl)-1,2,3,6-tetra-O-acetyl-D-glucopyranose can be obtained after esterification with acetic anhydride with anhydrous sodium acetate as a catalyst [18-21] with or without solvent [21, 22] at room temperature [20], at 50-60°C [21], boiling, or under microwave heating [18, 19, 22]. Lactose acetates were applied as intermediates for glycosyl azides in the presence of a variety of Lewis acids (SnCl₄, TiCl₄, BF₃.OEt₂, TMSOTf). They are also intermediates for an anomeric azidation of per-O-acetylated [23], for aminoethyl glycosides of cell-surface carbohydrates [24], or transesterification [22].

However, some of lactose acetate's functional properties and biological activity have not been revealed and studied in detail yet. Therefore, the microwave-assisted synthesis of lactose esters with acetic anhydride with specific functional characteristics remains challenging. This study aimed at a "green" synthesis of lactose octaacetate by microwave irradiation and to establish its functional characteristics, antimicrobial and antifungal properties, and cytotoxicity, and antiviral activities.

2. Materials and Methods

2.1. Materials.

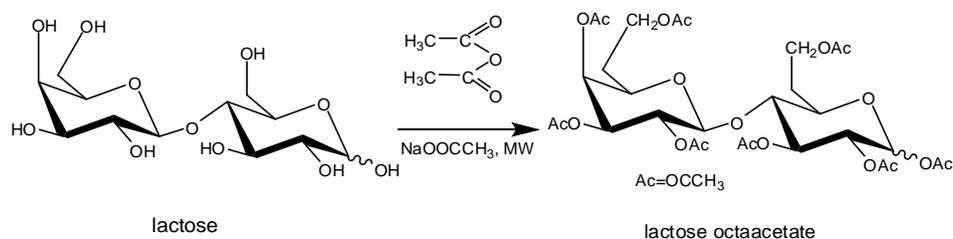
All reagents were purchased from Sigma Aldrich (Germany), and they were used as received. Synthesis of lactose octaacetate was conducted using D(+)-lactose monohydrate (Fluka Biochemica, Steinheim), acetic anhydride (Sigma Aldrich, USA), anhydrous sodium

acetate (Merck, Germany), and 95% (v/v) ethanol (Merck, Germany) for recrystallization of esters.

2.2. Synthesis and characterization of lactose acetates.

2.2.1. Synthesis of lactose octaacetate.

Octa-O-acetyl-D-lactose was synthesized as 10.0 g (0.029 mol) D-(+)-lactose monohydrate was mixed with 30 cm³ (0.27 mol) acetic anhydride in a round-bottom flask with 3.0 g (0.036 mol). Sodium acetate was used as a catalyst, and the reaction was performed under microwave irradiation (700 W for 10, 15, 20, and 30 minutes) (Scheme 1). The sample was then poured into 200 cm³ distilled water with ice cubes, stirred, and left at 4 °C for 12 hours. Lactose ester was precipitated as a white solid. Then the lactose ester was filtered under vacuum, and a clean-up procedure was performed with washing with distilled water. Lactose acetate was further purified by recrystallizing with 95% ethanol and then distilled water. Lactose acetate was dried in a vacuum oven to the constant weight. The lactose octaacetate was further characterized.



Scheme 1. One-pot synthesis of lactose octaacetate under microwave irradiation.

2.2.2. Melting point.

Melting points of lactose esters were measured with a Kofler hot stage microscope (Reichert, Austria).

2.2.3. Degree of substitution.

The percentage of acetylation (%) and the degree of substitution (DS) were determined by titration [25]. Octa-O-acetyl-D-lactose (0.5 g) was weighed in a 100 mL flask, and 30 mL of 75 % v/v ethanol was added. The sample was stirred on a magnetic stirrer for 15 minutes. Then was added 25 mL 0.5 M KOH and the sample was stirred on a magnetic stirrer for 2 hours. The excess of alkali was back-titrated with 0.5 mol/L HCl using indicator phenolphthalein. The blank samples and duplicates were analyzed similarly. The acetyl percentage was calculated.

2.2.4. Angle of optical rotation.

The optical rotation of 0.01% methanol solutions of lactose octaacetate was determined on an automated polarimeter in a tube 1 dm long with a volume of 10 mL using a sodium lamp as a light source.

2.2.5. Thin-layer chromatography (TLC).

The thin-layer chromatography was performed on a silica gel Kieselgel 60 F₂₅₄ plates (Merck, Germany) with eluent with composition ethyl acetate/methanol/water 17:2:1 (v/v/v). The spots appeared after spraying the plates with 10 % (v/v) H₂SO₄ methanol solution. The spots were detected after heating in an oven at 120 °C for 5 min [26].

2.2.6. UV spectroscopy.

Lactose acetates in concentration 1 mg/mL were prepared in the solvents with different polarities: methanol, ethanol, acetonitrile, dimethylformamide (DMFA), and dimethyl sulfoxide (DMSO). The UV spectra were collected on a UV-30 SCAN spectrophotometer in the wavelength range from 190–400 nm [10].

2.2.7. FT-IR spectroscopy.

The infrared spectra were recorded in KBr pellets on a Nicolet FT-IR Avatar Nicolet (Thermo Scientific, USA) spectrometer. The absorption was reported in wavenumbers (cm⁻¹) in the frequency range of 4000–400 cm⁻¹. Each spectrum was recorded after 132 scans.

2.2.8. Nuclear magnetic resonance spectroscopy (NMR).

The ¹H and ¹³C NMR spectra of Octa-O-acetyl-D-lactose were recorded on a Bruker Advance III 500 MHz spectrometer (Bruker BioSpin, Wissembourg, France) operating at 151 MHz using CDCl₃. Lactose acetate (20 mg) was dissolved in 0.6 mL CDCl₃, as a solvent, and spectra were collected. All chemical shifts were reported in ppm with reference to TMS.

¹H NMR (500 MHz, CDCl₃) δ 5.62 – 5.41 (m, 3H), 5.08 (dd, J = 39.8, 18.8 Hz, 7H), 5.04 (d, J = 9.7 Hz, 2H), 4.86 (dddd, J = 46.1, 36.3, 16.8, 8.2 Hz, 18H), 4.49 (ddd, J = 61.7, 46.4, 8.1 Hz, 6H), 4.20 (d, J = 10.9 Hz, 3H), 4.06 (d, J = 11.0 Hz, 5H), 3.98 – 3.80 (m, 20H), 3.81 (d, J = 7.7 Hz, 4H), 4.32 – 2.82 (m, 55H), 4.32 – 2.82 (m, 55H), 3.84 – 3.41 (m, 21H), 3.40 (s, 2H), 2.64 – 0.29 (m, 128H).

¹³C NMR (126 MHz, CDCl₃) δ 173.22, 170.87, 170.58, 170.45, 170.29, 170.25, 170.19, 170.05, 169.99, 169.86, 169.77, 169.55, 169.36, 169.31, 168.95, 168.91, 101.53 (C1 β-D-Galp), 100.74 (C1 β-D-Galp), 100.52 (C1 α-Glc) 91.30, 81.50, 77.67, 77.41, 77.16, 75.41, 73.12, 72.82, 72.36, 72.24, 71.22, 71.10, 70.71, 70.61, 70.38, 70.29, 69.45, 68.85, 68.52, 66.85, 66.75, 62.21, 61.66, 60.96, 20.44, 20.40, 20.35, 20.29, 20.22, 20.13, 20.06.

2.3. Functional properties of lactose acetates.

2.3.1. Swelling properties.

The swelling properties of lactose acetates were evaluated [27]. Lactose acetates (100 mg) were hydrated with 10 mL of distilled water in a calibrated cylinder at 25 °C. After an 18 h equilibration, the bed volume was recorded and expressed as an mL/g sample.

2.3.2. Water holding capacity (WHC) and oil holding capacity (OHC).

The WHC and OHC of lactose acetates were evaluated in duplicate [28]. Lactose acetate (LA 10 s) (0.10 g) was added into a pre-weighed 50 mL centrifuge tube. Then 10 mL of deionized water or sunflower oil was added, respectively. After 12 h at 25 °C, the sample

was centrifuged at 3500 rpm for 15 min. The excess water and oil were discarded. The tubes were weighed and dried at 105°C to the constant weight.

2.3.3. Foam stability (FS) and foamability (FA).

FS and FA of lactose acetates were also evaluated as all experiments were performed in triplicate [29]. The aqueous dispersion of lactose acetates (20 mL) in 0.01% concentrations was placed in 50 mL stoppered graduated cylinders, and the initial height of each solution was checked. The solution was vigorously shaken for 1 min, and the foam height and the total height were measured in cm immediately. The foam heights at 1, 2, 3, 5, 10, 20, 30, and 60 min were recorded at 25 °C.

3. Antimicrobial activity.

3.1. Test microorganisms.

Eight Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus cereus*, *Bacillus amyloliquefaciens* strain 4BCL-YT, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 8632, *Enterococcus faecalis*, *Micrococcus luteus* strain 2YC-YT, *Curtobacterium flaccumfaciens* strain PM-YT), five Gram-negative bacteria (*Salmonella enteritidis*, *Klebsiella* sp., *Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027), yeasts (*Candida albicans* NBIMCC 74, *Saccharomyces cerevisiae*), and fungi (*Aspergillus niger* ATCC 1015, *Aspergillus flavus*, *Penicillium* sp., *Rhizopus* sp., *Fusarium moniliforme* ATCC 38932) from the collection of the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria, were selected for the antimicrobial test. The concentration of the viable cells and spores in the suspensions for inoculation was adjusted to $1.0 \cdot 10^5$ CFU mL (for fungal spores) and $1.0 \cdot 10^9$ CFU mL (for bacterial and yeast cells).

3.2. Culture media.

For the cultivation of the Gram-positive bacteria, Gram-negative bacteria, and yeasts were used Luria-Bertani glucose agar medium (LBG agar medium) prepared as previously described [30].

Malt extract agar (MEA) was used to cultivate the fungi. This media has consisted of 20 g malt extract, 20 g dextrose, 6 g peptone, and 15 g agar dissolved in 1 L of deionized water. The final pH was corrected to 5.5; the medium was sterilized by autoclaving at 121°C for 20 min.

3.3. Antimicrobial assay.

Methanol and DMSO were used as solvents to prepare the desired solutions 10 mg/mL of lactose acetate. The antimicrobial activity of samples was determined using the agar well diffusion method [30].

Lactose acetates and controls (60 µL) were placed into the agar wells in triplicates. Methanol and DMSO were used as controls, as positive controls were used Streptomycin, Ampicillin, and Nystatin. The antimicrobial activity of lactose acetates was evaluated by the diameter of the inhibition zones around the wells on the 24th and the 48th hour of incubation. The inhibition zones of 18 mm or more were listed as sensitive (strong inhibitory effect); with

zones from 12 to 18 mm - moderate inhibitory effect, with the inhibition zones up to 12 mm or completely missing - insignificant or low inhibitory effect.

4. Cytotoxic and antiviral activity.

4.1. Cells.

Madin-Darby bovine kidney (MDBK) cells and Human epithelial type 2 (HEp-2) cells from human laryngeal carcinoma were obtained from National Bank for Industrial Microorganisms and Cell Cultures, Sofia. Madin-Darby canine kidney MDCK cells (NBL-2; CCL-34) were purchased from ATCC, Manassas, USA. The three cell lines were grown in DMEM medium containing 10% fetal bovine serum (FBS) (Gibco BRL, USA), with 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL) in a CO₂ incubator (HERA cell 150, Heraeus, Germany) at 37°C/5% CO₂.

4.2. Viruses.

Most of the viruses, except *Herpes simplex* virus type 1 (HSV-1), used in the current research, were collected from the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria). Only *Herpes simplex* virus type 1 (HSV-1), Victoria strain was the National Center of Infectious and Parasitic Diseases, Sofia.

Coxsackievirus B1 (Connecticut 5 strain, CVB1) was propagated in HEp-2 cells (maintenance solution DMEM (Gibco, BRL) with 10 mmol/l HEPES, 0,5% FBS, penicillin 100 IU/mL and streptomycin 100 mg/mL); infectious titer - 10^{6.5} CCID₅₀/mL.

PV-1 (strain LSc-2ab) was replicated in HEp-2 cells (maintenance solution DMEM (Gibco, BRL) with 10 mmol/l HEPES, 0.5% FBS, penicillin 100 IU/mL and streptomycin 100 mg/mL); infectious titer - 10⁷ CCID₅₀/mL.

Herpes simplex virus type 1(HSV-1) virus was reproduced in monolayer MDBK cells in a maintenance solution DMEM Gibco BRL, Paisley, Scotland, UK, plus 0.5% FBS. An infectious titer of stock virus was 10⁷ CCID₅₀/mL.

Influenza virus A/Panama/2007/99/H3N2 - (IAV/H3N2) was obtained from allantoic fluids of virus-inoculated 10-days-embryonated eggs, incubated at 37°C; infectious virus titer determined as 10^{6.0} CCID₅₀/mL.

4.3. Cytotoxicity assay.

Confluent monolayer cell cultures MDBK, HEp-2, or MDCK in a 96-well plate was treated with a culture medium containing no (untreated control) or increased concentrations of the compounds. The cells were incubated at 37°C and 5% CO₂ for 48 h. After drug treatment, the viability of the cells was measured using a neutral red uptake assay and ELISA reader at wavelength 540 nm.

4.4. Antiviral activity assay.

Cytopathic effect (CPE) inhibition test was performed and was calculated, as previously described [31]. The 50% inhibitory concentration (IC₅₀) was defined as the concentration inhibited 50% of viral replication compared to the virus control. The selectivity index (SI) was calculated from the ratio CC₅₀/IC₅₀.

4.5. Virucidal assay.

The contact samples of 1 mL containing HSV-1 (105 CCID50) and tested compounds in their maximal tolerate concentration (MTC) in a 1:1 ratio. The samples were stored at room temperature for different time intervals (15, 30, 60, 90, and 120 min). Then, the infectious viral titer of all samples was determined by endpoint dilution titration. The results were compared to those from virus control - an equal volume of virus suspension and maintenance medium, incubated for the same time intervals, and $\Delta\log$ was calculated.

4.6. Virus attachment assay.

The cell monolayers of MDBK, or HEp-2 cells in 24-well cell culture plates (pre-chilled at 4°C) were inoculated with 104 CCID50 of HSV-1, CBV1, or PV-1 at 4°C and treated in parallel with maximal tolerate concentration (MTC) of the compounds [31].

4.7. Pretreatment of MDBK cells.

Monolayers of MDBK cells in 24-well cell culture plates (CELLSTAR, Greiner Bio-One) (2×10^6 cells per well) were tested for 15, 30, 60, 90, and 120 min at a concentration of MTC of lactose octaacetate, according to the procedure [31]. The results were compared to those from virus control - cells infected with the virus but not pre-treated with the extract. Then $\Delta\log$ s were determined.

5. Results and Discussion

5.1. Synthesis and characterization of lactose acetates.

The results from the microwave-assisted synthesis of lactose esters are summarized in Table 1. Moreover, the results from the green method of synthesis were compared with conventional acetylation. All lactose esters present white to faint yellow powder substances slightly bitter taste. Bitter tastes for sugar acetates were also reported [32-34]. In our previous studies, the bitter taste was reported for sucrose and inulin acetates [4, 10]. The full substitution of hydroxyl groups and its modification in acetyl esters alter sweet sugar to bitter acetyl derivatives.

Table 1. Characterization of lactose acetates with molar ratio (1:10:5).

Sample	Conditions	Time, min	Yield, %	Melting point, °C	$[\alpha]_D^{25}$ (c=0.01, CH ₃ OH)	Degree of substitution
LA 1	Microwave irradiation, 700 W	10	78	89.8 – 90.8	-28	3.71
LA 2	Microwave irradiation, 700 W	15	91	90.1 – 91.1	-27	3.27
LA 3	Microwave irradiation, 700 W	20	91	89.9 – 90.9	-27	3.21
LA 4	Microwave irradiation, 700 W	30	39	93.8 – 94.8	-28	3.54
LA C	Conventional	60	85	90.5 – 91.5	-27	3.37

The highest yield (91%) was obtained for 15-20 min with microwave-assisted irradiation. In our study, the yield of lactose octaacetates was higher than reported yields using iodine, pyridine, FeCl₃ [22] and comparable with Patil *et al.* [18] for 25 sec at MW conditions (Yield 75-89%). Moreover, the acetylation of 50% was obtained only for 10 min, which reveals the advantage of microwave irradiation. It reduces acetylation time more than 5 times as yield remains constant compared to conventional esterification. The melting points of lactose acetates were in the range of 89-91.5°C. The substitution of hydroxyl groups with acetyl

residues lowers the melting point of lactose esters. Similar trends were observed in other studies for acetates of lactose, sucrose, and inulin [4, 10, 18, 34]—optical rotation changes from +52.3° for lactose to negative values (Table 1). A negative angle of optical rotation but with smaller values was also reported in the literature [18].

The quantitative analysis of lactose esters was performed by TLC analysis (Figure 1). The results showed that all lactose esters had $R_f = 0.74$, which was comparable with those of other sugar esters – sucrooctaacetate $R_f = 0.76$.

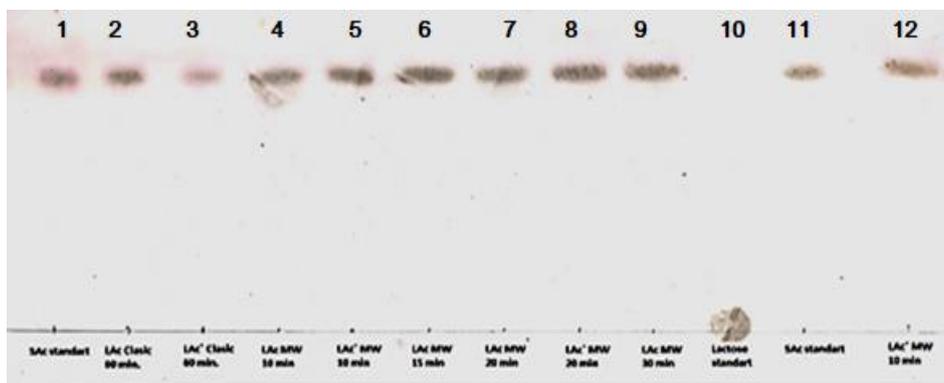
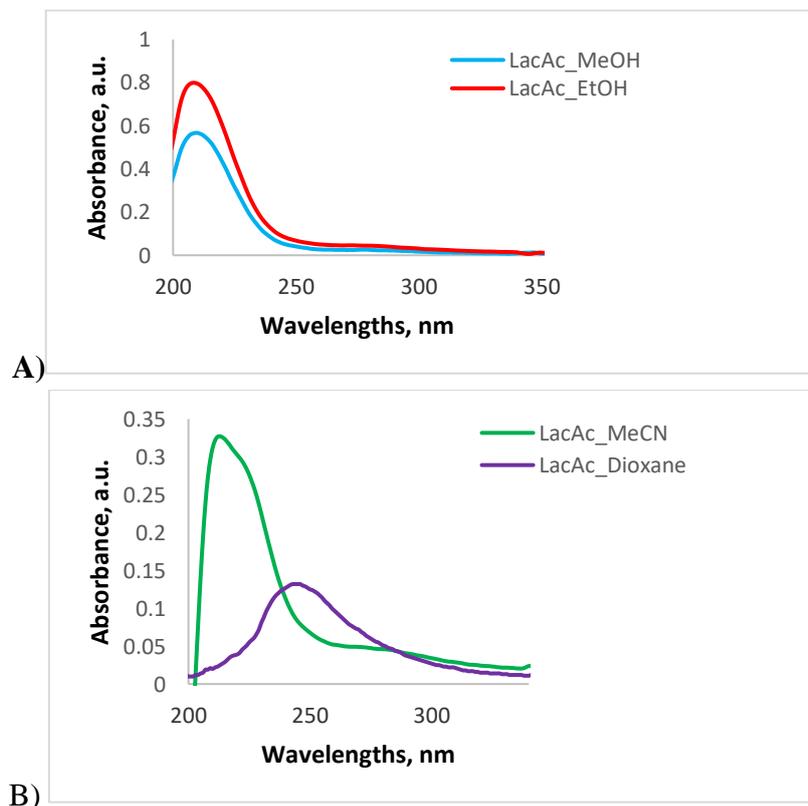


Figure 1. TLC chromatograms of lactose acetates synthesized under different conditions, where 1. SAC-sucrooctaacetate (standard), 10. lactose and 2-12. lactose octaacetate in ethyl acetate/methanol/water 17:2:1 (v/v/v) as mobile phase and 10% H₂SO₄ as a detecting reagent.

5.2. UV Spectroscopy.

In Figure 2, the UV spectra of lactose acetates were presented in the wavelengths range from 190 to 400 nm. The absorption properties of investigation compounds in different polarity solvents (methanol, ethanol, acetonitriledioxane, dimethylformamide, dimethyl sulfoxide, etc.) were summarized in Table 2.



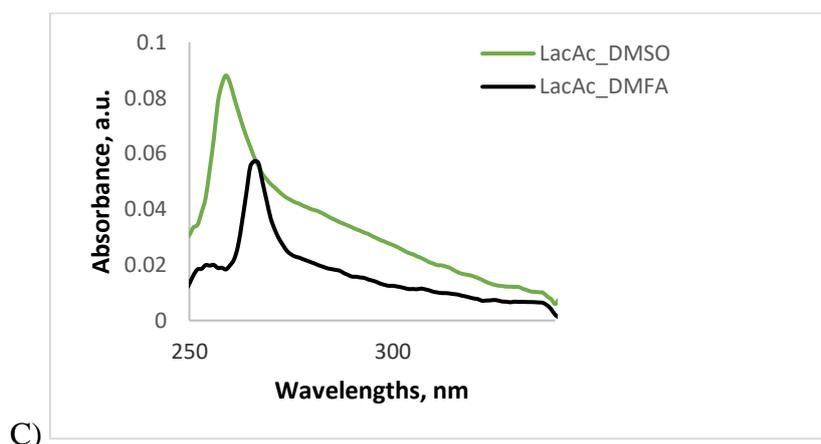


Figure 2. UV spectra of lactose octaacetate (1 mg/mL) in different solvents: **A)** methanol and absolute ethanol, **B)** acetonitrile and dioxane, **C)** N,N-dimethylformamide (DMFA), and dimethyl sulfoxide (DMSO).

Table 2. The absorption properties of lactose octaacetate in different solvents.

No	Solvents	Polarity index [35]	ΔF , (ϵ , n^2) [36]	λ_{max} (A) (nm)	ϵ , ($L \cdot mol^{-1} \cdot cm^{-1}$)
Proton donating solvents					
1.	Methanol	5.1	0.2887	209	0.5670
2.	Ethanol	5.2	0.1548	208	0.7991
Proton accepting solvents					
3.	Dioxane	4.8	0.0204	245	0.1320
4.	Acetonitrile	5.8	0.3045	213 221 shoulder	0.3272 0.2991
5.	DMFA	6.4	0.2743	266	0.0572
6.	DMSO	7.2	0.2634	259	0.0880

DMFA - N,N-dimethylformamide; DMSO - dimethyl sulfoxide.

The absorption maximum in both protons donating solvents methanol and ethanol was detected at 209 nm and 210 nm, respectively. In the acetonitrile spectrum of lactose acetates, the maximum absorption band was found at 213 nm with a shoulder at 221 nm. Stagner *et al.* [34] reported an absorption maximum of sucrose octaacetate in mixed solvent acetonitrile-water (30:70) at 210 nm. In solvent 1,4-dioxane the absorption maximum was observed at 245 nm and DMF and DMSO – at 266 nm and 259 nm, respectively. Petkova *et al.* [10] investigated inulin acetate esters and reported absorption maxima in solvents N, N-dimethylformamide, and dimethylsulfoxide at 266 nm and 257.5 nm, respectively. This is the first study where the absorption maximum of lactose octaacetates was evaluated.

5.3. FT-IR spectroscopy.

FT-IR spectroscopy octa-O-acetyl-D-lactose synthesized by microwave irradiation was used to identify functional groups. Acetylation of lactose was confirmed by FTIR spectra (Figure 3).

The broadband at 3320 cm^{-1} typical for the stretching vibrations of free OH groups in lactose were not observed after esterification. This is due to the fact that OH was replaced with acetyl ($-\text{COCH}_3$) residues. In the FTIR spectrum of lactose acetates, a new band at 1752 cm^{-1} was observed that was due to stretching vibration of $\text{C}=\text{O}$ groups from acetyl ester (Figure 3). New bands also appeared at 1371 cm^{-1} assigned with the bending of $\text{C}-\text{H}$ from CH_3 and $-\text{C}-\text{O}$ stretching. FTIR spectrum of lactose acetate contained other typical bands for lactose at 2985 cm^{-1} that was assigned to CH_2 , wagging CH_2 at 1436 cm^{-1} and stretching vibration of $\text{C}-\text{O}$ at 1047 cm^{-1} . In addition, the acetyl ($-\text{COCH}_3$) groups were also observed at 1220 cm^{-1} . The

bands at 952 cm^{-1} confirmed that this ester contained β -D-galactopyranosyl residues linked by $1\rightarrow4$ glycoside bonds. All these findings confirmed the successful acetylation of lactose. Most of the bands were in accordance with reported data for lactose and sucrose acetate [10, 24, 37, 38].

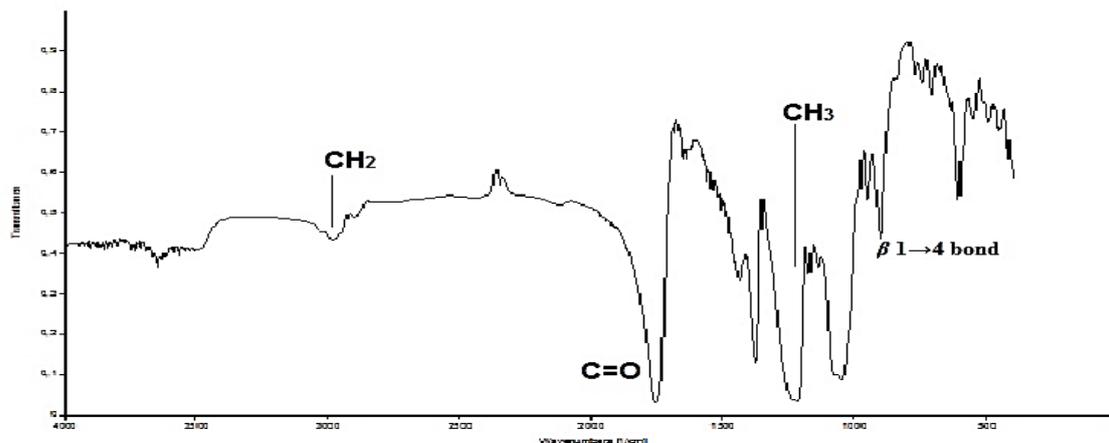


Figure 3. FTIR spectrum of octa-O-acetyl-D-lactose (LA1) synthesized by microwave irradiation.

5.4. Nuclear magnetic resonance spectroscopy (NMR).

The NMR spectra approve the structure of obtained lactose esters.

NMR spectra showed that lactose acetates consisted of a mixture of 2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-($1\rightarrow4$)-1,2,6-tri-O-acetyl α,β -D-glucopyranose, $\alpha:\beta = \sim 1:2.2$.

^1H NMR spectrum of lactose acetate contained chemical shifts typical for a methyl side chain of acetyl groups (COCH_3) that was found at ~ 2 ppm. Chemical shifts for acetyl carbonyl was observed at δ 173.22, 170.87, 170.58, 170.45, 170.29, 170.25, 170.19, 170.05, 169.99, 169.86, 169.77, 169.55, 169.36, 169.31, 168.95, 168.91 ppm. All the methyl carbons from the acetyl residue appeared at δ 20 ppm (COCH_3). The chemical shifts relevant to carbon atoms of the lactose (glucose and galactose residues) were found in the range of 62.70 to 101.53 ppm. The shift at 101.53 ppm typical for C_1 from galactose that was involved in the linkage β -($1\rightarrow4$)-D-glucopyranose residue were found in an anomeric region (Figure 4). Similar shifts were reported for lactose acetates synthesized by classical method and microwave methods [19, 25]. Reported by shifts were near to Peng [19]: ^{13}C NMR (CDCl_3 , α anomer): δ 101.9 (C-1II), 89.2 (C-1I), 82.1 (C-4I), 71.5 (C-5II), 70.8 (C-3II), 70.5 (C-2I), 69.6 (C-5I), 69.5 (C-3I), 68.7 (C-2II), 66.8 (C-4II), 62.1 (C-6I), 61.8 (C-6II); ^{13}C NMR (CDCl_3 , β anomer): δ 102.0 (C-1II), 91.6 (C-1I), 82.1 (C-4I), 73.3 (C-3I), 72.6 (C-5I), 71.5 (C-5II), 71.2 (C-2I), 70.8 (C-3II), 68.6 (C-2II), 66.8 (C-4II), 62.2 (C-6I), 61.8 (C-6II).

5.5. Functional properties.

5.5.1. Swelling properties.

Lactose acetates demonstrated better swelling properties (2.82 mL water/g sample) in comparison to FOSs acetates (1.37 mL water/g sample) and inulin acetates with longer chains [10, 29]. The number of acetylated hydroxyl groups influenced on swelling properties of carbohydrate acetylated ester. The swelling properties decreased in the following order monosaccharide > oligosaccharide > polysaccharide acetates [19].

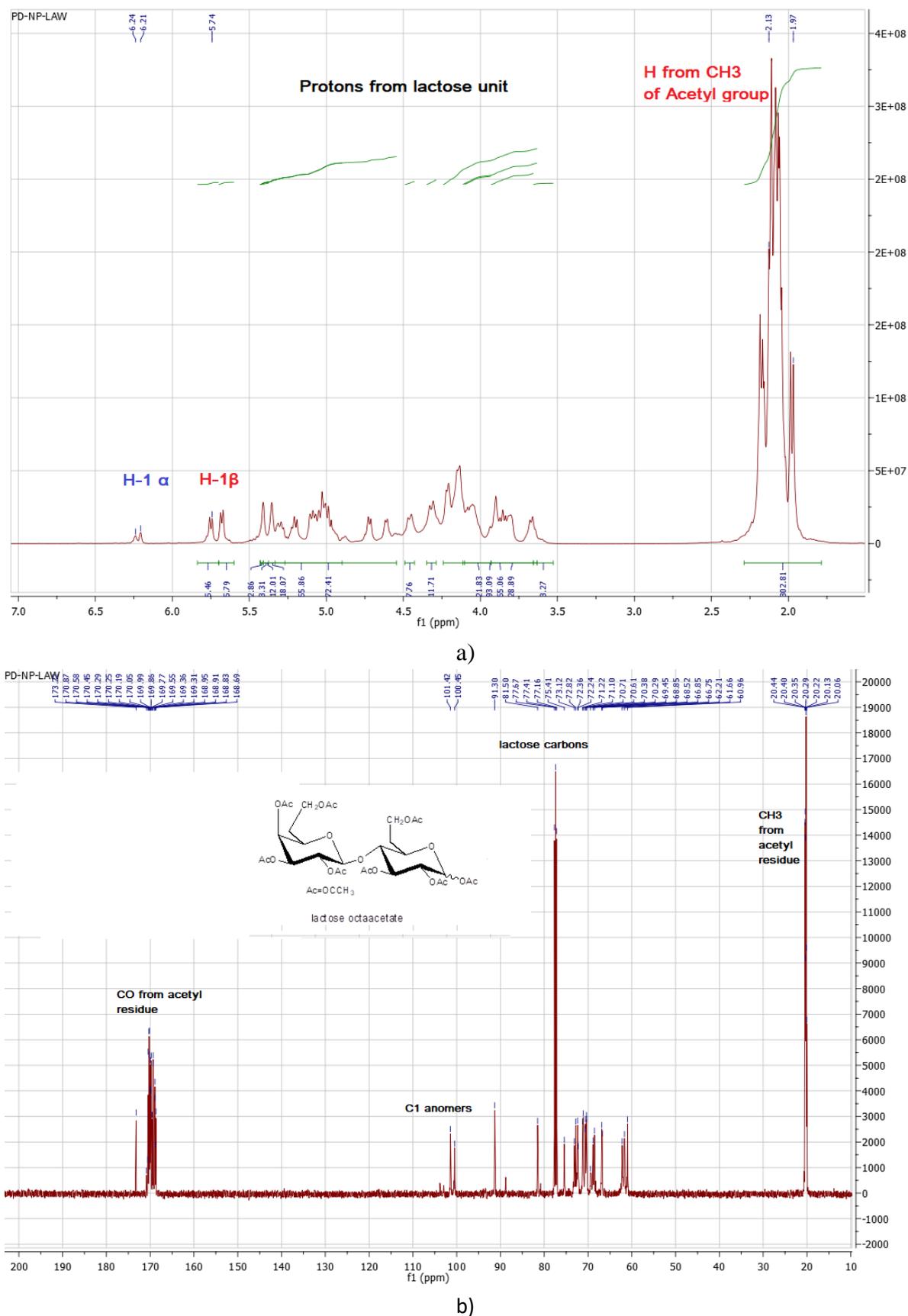


Figure 4. (a) ^1H NMR and (b) ^{13}C NMR spectra of octa-O-acetyl-D-lactose synthesized by microwave irradiation.

5.5.2. WHC and OHC.

Water holding and oil holding properties caused an effect on functional and sensory properties. Therefore, these features enlarge the application of modified carbohydrates in different industries. Until now, many studies about the evaluation of WHC and OHC of lactose octaacetates were not provided, and there is the absence of information about these functional properties. . Petkova *et al.* [29] investigated the influence of acetylation of FOSs with different DP and found that brought about the increase of their OHC. The OHC of lactose acetate was higher than their WHC and reached 3.4 g oil/g sample (Figure 5). Functional properties of lactose esters were compared with β -D-glucose pentaacetates (Figure 5).

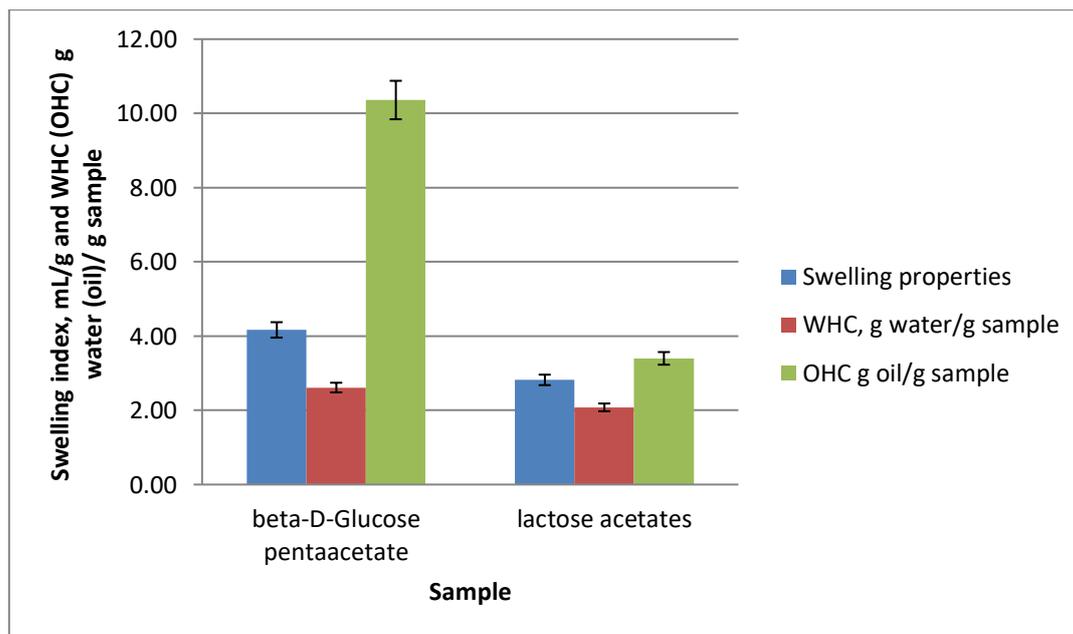


Figure 5. Swelling properties, water holding, and oil holding capacity of lactose acetate (LA 1) synthesized under microwave irradiation.

Lactose acetates and β -D-glucose pentaacetates demonstrated similar small values of WHC that did not exceed 2.5 g water/g sample. However, β -D-glucose pentaacetates retained three times more oil than lactose esters (Figure 5). OHC of lactose acetates was near that of inulin and starch acetates [10, 39]. The higher values of OHC in acetylated carbohydrates could be explained with more acetylated residues. The complete substitution of hydrophilic OH groups in the lactose backbone with acetyl residues explained these properties. It was demonstrated that acetylation improved the water and oil holding capacities as a result of the incorporation of acetyl groups on the starch molecules which led to better binding capacity [39]. In different starches, their water and oil absorption capacities increase with the increased acetic anhydride concentration in carbohydrate chains [39,40]. The current study dealing with the OHC of lactose acetates demonstrated their potential use in pharmaceutical and food formulations.

5.5.3. Foaming properties of lactose acetates.

The foaming properties of modified carbohydrates with the short and long-chained fatty acid ester were reported [4, 5, 10, 16, 21, 42]. However, until now, any data for the potential of lactose acetates as a foaming agent were not available. FA values for lactose acetate at a concentration of 0.1% are 95%. The FS of the obtained lactose acetates was estimated by

measuring the foam height of 1–60 min (Figures 6). For the foam obtained with 0.1% lactose acetate, FS decreases by more than 80% for the first 5 minutes, while the reduction of the foam for the next 5 minutes is 20%. In comparison, the FS of octa-O-acetylsucrose (0.1 g/L) does not exceed 50–55% in 30 minutes [4]. The results demonstrate the formation of lactose acetate foam with low stability (10–20 %) in the studied concentration (0.1%). It was reported that the foam height drops with the increase of the fatty acid carbon number and the presence of diesters in the preparation [43]. Some long-chained raffinose esters at concentration 0.2% w/w showed superior foamability (78.11 %) [41] near to our results with lactose acetates. Some authors explained low foamability with the smallest alkyl moieties [41]. In our case, low foam stability could be explained because lactose acetates were polyesters and contained short-chain hydrophobic residues. The foams formed with lactose acetates were stable until 5 min at concentration 0.1 %. Additional experiments should be provided to test the influence of concentration on foam stability of lactose esters.

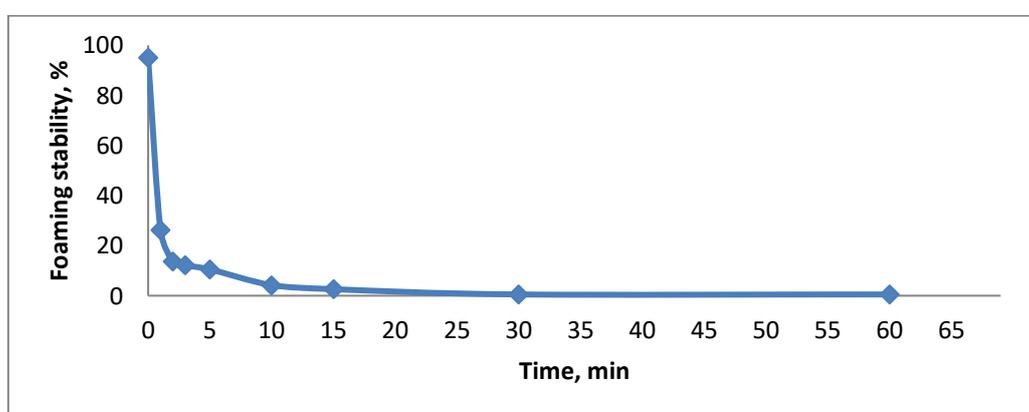


Figure 6. Foaming stability of lactose acetate (LA1).

5.6. Antimicrobial activity.

This study presents the first report for the antimicrobial activity of lactose acetates analyzed against twenty microorganisms. The results from the antimicrobial activity of lactose acetates (LA1) are summarized in Table 3. Its activity was comparable with the control antibiotic Nystatin 40 µg/mL. From the obtained results, it was found that lactose acetates in the tested concentration did not inhibit the growth of Gram-positive and Gram-negative bacteria and yeasts. However, lactose acetylated esters possessed antifungal activity against *Aspergillus niger* ATCC 1015, *Penicillium sp.*, *Rhizopus sp.*, and *Fusarium moniliforme* ATCC 38932 (Table 3). Against *Aspergillus flavus* these esters were inactive. The obtained results found that lactose acetates showed moderate activity *Rhizopus sp.* and *Fusarium moniliforme* ATCC 38932. Compared to our previous research for sucrose octatacetates, lactose acetates demonstrated similar antimicrobial activity [4]. Similar to inulin acetates and inulin derivatives modified with Schiff bases [44], lactose acetates demonstrated mainly antifungal activity.

Table 3. Antimicrobial activity of lactose octaacetes (LA) expressed as the diameter of zones of inhibition in mm ($d_{disc} = 6$ mm).

Test microorganism	Lactose esters		Solvents		Positive controls		
	LA in MeOH	LA in DMSO	DMSO	MeO H	Streptomycin 30 µg/mL	Ampicillin 10 mg/mL	Nystatin 40 µg/mL
Gram (+) bacteria							
<i>B. subtilis</i> ATCC 6633	-	-	-	-	28***	21	n/a
<i>B. cereus</i>	-	-	-	-	25***	40	n/a
<i>B. amyloliquefaciens</i> strain	-	-	-	-	24***	40	n/a

Test microorganism	Lactose esters		Solvents		Positive controls		
	LA in MeOH	LA in DMSO	DMSO	MeO H	Streptomycin 30 µg/mL	Ampicillin 10 mg/mL	Nystatin 40 µg/mL
4BCL-YT							
<i>S. aureus</i> ATCC 25923	-	-	-	-	25***	40	n/a
<i>L. monocytogenes</i> ATCC 8632	-	-	-	-	23***	33	n/a
<i>E. faecalis</i>	-	-	-	-	22***	38	n/a
<i>M. luteus</i> strain 2YC-YT	-	-	-	-	31***	40	n/a
<i>C. flaccumfaciens</i> strain PM-YT	-	-	-	-	35***	38	n/a
Gram (-) bacteria							
<i>S. enteritidis</i>	-	-	-	-	18**	38	n/a
<i>Klebsiella</i> sp.	-	-	-	-	18**	14**	n/a
<i>E. coli</i> ATCC 8739	-	-	-	-	26***	40	n/a
<i>P. vulgaris</i> ATCC 6380	-	-	-	-	24***	38	n/a
<i>Ps. aeruginosa</i> ATCC 9027	-	-	-	-	23***	-	n/a
Yeasts							
<i>C. albicans</i> NBIMCC 74	-	-	-	-	n/a	n/a	13**
<i>S. cerevisiae</i>	-	-	-	-	n/a	n/a	-
Fungi							
<i>A. niger</i> ATCC 1015	10*	10*	-	-	n/a	n/a	-
<i>A. flavus</i>	-	-	-	-	n/a	n/a	8*
<i>Penicillium</i> sp.	10*	10*	-	-	n/a	n/a	9*
<i>Rhizopus</i> sp.	10*	15**	-	-	n/a	n/a	10*
<i>F. moniliforme</i> ATCC 38932	10*	10*	-	-	n/a	n/a	-

***- strong inhibitory effect, **- moderate inhibitory effect, * slight inhibitory effect, - low inhibitory effect; n/a – not applicable.

Moreover, acetylated saccharides with pyranose structures have higher antimicrobial and antifungal activities than acetylated saccharides with furanose structures [20]. In comparison to other lactose derivatives, it was demonstrated that O-β-D-galactopyranosyl-(1→4)-N-alkyl-(3-sulfopropyl)-D-glucosamine hydrochloride demonstrated antibacterial and fungistatic activity (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*), yeasts (*Candida albicans*) and filamentous fungi (*Fusarium graminearum*, *F. avenaceum*, *F. oxysporum*, *F. culmorum*, *F. equiseti*, *Alternaria alternata* and *Botrytis cinerea*) [42]. It was reported that some lactose esters with long-chain fatty acids as lactose palmitoleate and lactose nervonate showed antimicrobial activity versus eight pathogenic species belonging to Gram-positive, Gram-negative microorganisms, and fungi [45]. It was demonstrated that lactose esters esterified with saturated fatty acid as decanoic and lauric acids showed greater antimicrobial properties than lactose esters of octanoate and myristate against Gram-positive bacteria [46]. In our case, lactose octaacetates were inactive against Gram-positive, Gram-negative, and yeast but demonstrated antifungal activity.

5.7. Cytotoxicity and antiviral effect of lactose acetates.

Until now, any information about cytotoxicity and antiviral activity of lactose acetate was not found. Research deals with unsaturated fatty acid esters with lactose that revealed its cytotoxicity characterizations, their permeability enhancing activity was demonstrated using intestinal Caco-2 monolayer through transepithelial electrical resistance (TEER) and permeability [45]. Antiviral activity against some of the studies viruses was reported for polysaccharides – carrageenans [47]. Therefore, this is the first detailed study for cytotoxicity and antiviral activity of lactose octaacetates.

The cytotoxicity of synthesized lactose acetate was determined by three cell lines, MDBK, MDCK, and Hep-2, which are then the basis for conducting antiviral experiments (Table 4). The results showed the lowest toxicity of lactose acetates on the MDBK cell line and

close cytotoxicity in Hep-2, whereas, in the MDCK cells, it demonstrates more than twice higher toxicity. Testing the antiviral activity of synthesized lactose acetate revealed inhibition of replication of PV-1 with a selective index of 2.4, but it was not found any specific antiviral effect against CVB1, HSV-1, and IAV/H3N2 replication (Table 4).

Table 4. Cytotoxic and antiviral activity of lactose acetates.

Cells and Viruses	Cytotoxicity (µg/mL)		Antiviral activity	
	CC50	MTC	IC50 (µg/mL)	SI
Hep-2	533	250	-	-
MDCK	688	200	-	-
MDBK	1100	100	-	-
PV-1	-	-	215	2.4
CVB1	-	-	-	-
HSV-1	-	-	-	-
IAV/H3N2	-	-	-	-

The invested lactose octaacetate also showed antiviral activity against extracellular virions of HSV-1 virions. The effect was the strongest one (Table 5) in the HSV-1 virions, with the effect still accounting for 15 min of exposure ($\Delta\log = 1.25$) maintained with this value until 60 min ($\Delta\log = 1.5$).

Table 5. The viricidal activity of lactose acetate on HSV-1 virions.

$\Delta\log$				
15 min	30 min	60 min	90 min	120 min
1.25	1.25	1.50	1.50	1.50

After determining the effect of lactose acetate on extracellular virions and replication of intracellular viruses, its further effect was monitored on the stage of adsorption of the virus to the respective sensitive cells. The adsorption inhibition on all three viruses occurred with approximately the same decrease in viral titers. At 15 min the effect was weak in all three viruses; at 30 min it became significant in HSV-1 ($\Delta\log = 1.25$), while in the other two viruses, it still had relatively low values, and after 45 min the adsorption of all three viruses was significantly suppressed, with the strongest effect observed at SVB1 at 60 min ($\Delta\log = 1.4$) (Table 6).

Table 6. Effect of lactose acetates on the viral adsorption.

Virus	$\Delta\log$			
	15 min	30 min	45 min	60 min
HSV-1	1.25	1.25	2.75	2.75
PV-1	1.0	1.0	2.0	2.0
CVB1	0.0	0.4	1.0	1.4

The protective effect of lactose acetates was also examined for virus-free cells and to evaluate their activity to which it could protect cells from viral invasion. The model to use MDBK cells was selected due to the fact that lactose acetates showed the lowest cytotoxicity. The cells treated with the lactose acetates had a significant decrease in infectious viral titers. At the first studied time interval - 15 min, this decrease was with $\Delta\log = 1.5$ and remained with such a value for up to 120 minutes (Table 7).

Table 7. Protective effect of lactose acetate on uninfected cells with subsequent herpes infection.

$\Delta\log$				
15 min	30 min	60 min	90 min	120 min
1.5	1.5	1.5	1.5	2.75

6. Conclusions

This research showed the efficiency of microwave irradiation for rapid and effective synthesis of lactose acetates with acetic anhydride, sodium acetate and the lack of solvent media only for 10 min-20 min. The structure of the resulting esters was confirmed by FT-IR and NMR studies. To the best of our knowledge, the antimicrobial and antiviral activity of lactose esters was evaluated. Their antifungal potential against some plant and food-borne pathogens was demonstrated, as well as their functional properties. As a result of its cytotoxicity, antiviral and antifungal activities, lactose esters should be successfully applied for cosmetic purposes.

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Conflicts of Interest

The authors declare no conflict of interest.

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