

Lithuanian Royal jelly: quality assessment, biological activity and quantitative determination of *trans*-10-hydroxy-2-decenoic acid in various solvents

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Royal jelly is a bee product with a high commercial interest due to its exceptional biological properties and unique composition. In this study, we evaluated the quality of Lithuanian Royal jelly, including the water content, antioxidant activity, amount of polyphenolic compounds and pH. The results indicated that Lithuanian Royal jelly had the values of pH, water content as well as polyphenols that were comparable to the data already published. In the study, we also evaluated the impact of various solvents on the amount of 10-hydroxy-2-decenoic acid (10-HDA), the main fatty acid, naturally occurring only in Royal jelly and which is the quality marker, in solutions obtaining the amount using the HPLC method. According to solubility tests performed, 10-HDA from Royal jelly is best soluble in ethanol of various concentrations, yet in order to be used for exceptional Royal jelly antibacterial and many other biological properties it was important to increase the solubility in aqueous solutions. Addition of β -cyclodextrin has increased the amount of 10-HDA in solution up to the level of ethanol solutions. The antioxidant and antibacterial tests have demonstrated that Lithuanian Royal jelly exhibits positive effects against free radicals, as well as mostly occurring bacterial strains, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*. As the conclusion of this study, we can assume that the quality assessment methods chosen for this study, are significant for standardizing the procedures for further studies.

Keywords: Royal jelly, 10-HDA, antibacterial, antiradical, antioxidant, HPLC/DAD, DPPH• assay

INTRODUCTION

Royal jelly (RJ) is a gelatinous, white-with-pearl-shade substance produced by worker bees, with a unique smell and an acid taste [1]. Commercial interest in RJ is high because of its exceptional bi-

ological properties that are applied in many areas, from medication or food to cosmetics and others [2]. The unique and chemically interesting composition of RJ consists of lipids, proteins and fatty acids [3].

Fatty acids possess the highest biological effect, and the main fatty acid, with various bioactivity properties, is *trans*-10-hydroxy-2-decenoic acid

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(10-HDA) [3]. In nature, 10-hydroxy-2-decenoic acid (10-HDA), an important unsaturated fatty acid, is only available in Royal jelly. The composition of RJ varies, depending on the season and the location where the substance is harvested [4]; thus, the amount of 10-HDA in pure RJ may vary depending on its origin [5]. Since no other product produced by bees contains 10-HDA this fatty acid can be used as a marker for assessment of RJ in comparison with other bee products [5, 6]. Since 10-HDA is an important component of RJ, it is prudent to determine the amount of this acid in Lithuanian RJ, which can be achieved with high-performance liquid chromatography (HPLC) [37].

Reports suggest that RJ is a suitable candidate for modelling pharmaceutical products that exhibit antibacterial effects [7]. The antibiotic resistance of bacteria is a critical problem globally. Thus, alternative medicines to antibiotics are being sought to better control bacterial infections [7]; RJ is among these alternative products. It is believed that the antibacterial activity of RJ depends on the royalisin protein it contains. Royalisin is effective against gram-positive bacteria but not against gram-negative bacteria [8]. The antibacterial properties of RJ are associated with fatty acid 10-HDA [9].

Data on the biological properties of RJ and the chemical composition of its individual active compounds are scarce. Quality assessment of RJ is necessary to achieve a safe and purposeful application of RJ in food and pharmaceutical products. Some countries, such as Switzerland [15], Bulgaria, Brazil and Uruguay, developed national standards for quality assessment of RJ [2, 16]. However, Lithuanian Royal jelly has not been widely researched, therefore the aim of our original research is to perform the quality assessment of Lithuanian Royal jelly, evaluate its antibacterial and antioxidant activities, and to perform quantitative analysis of 10-HDA in various Royal jelly solutions.

EXPERIMENTAL

Six samples of RJ harvested in Lithuania in 2016–2018 were used for the study. Samples No. 1–2 were harvested in 2016, samples No. 3–4 in 2017 and samples No. 5–6 in 2018. The RJ was gathered in the apiary of beekeeper A. Bučius in the Kaunas

District. The samples of fresh RJ were packed in opaque plastic containers and frozen quickly after harvesting and kept frozen throughout the study.

Determination of pH values

The pH of the samples was determined with a pH-meter 766 Calimatic (Knick, Germany) at $20 \pm 1^\circ\text{C}$.

Water content in Royal jelly

The moisture content of RJ was determined with the gravimetric method, with a moisture analyser (KERN MLS, Germany).

Determination of antiradical activity

The antioxidant activity of RJ was determined with the DPPH• radical binding assay [18]. DPPH• is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored ethanol solutions. The use of DPPH• assay provides an easy and rapid way to evaluate antioxidants. To determine the antioxidant activity, a DPPH• solution of 0.1 mmol/l was made in 96% ethanol 24 h before the study. The solution was stored continually in a dark, cool place. In this method, 0.1 ml of the RJ is mixed with 2.9 ml of DPPH• solution; after 30 min the absorbance of the solution is measured with the spectrophotometer at 518 nm wavelength. Anti-radical activity was calculated according to the formula

Inactivated DPPH•content (percent) =

$$\left[\frac{A_o - A_t}{A_o} \right] \times 100,$$

where A_o is the absorption of DPPH• comparative solution, and A_t is the absorption of the test solution.

Determination of antibacterial activity of Lithuanian Royal jelly

The antibacterial properties of RJ were evaluated in vitro with the agar diffusion method using the Müller-Hinton agar (Mueller-Hinton agar Oxoid LTD (CM 0337), Basingstoke, Hampshire, England). In vitro studies were performed with the gram-positive and gram-negative reference bacteria strains *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC

25922) and *Pseudomonas aeruginosa* (ATCC 27853), and the clinical bacteria strains *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. In accordance with the standard approved by the Clinical and Laboratory Standards Institute, fluid Mueller-Hinton agar was prepared and poured into 10 cm diameter Petri dishes, 35 ml each, and left in the horizontal position to thicken. Bacterial strains were spread on the surface of the thickened medium, and six wells (7 mm diameter) were made in each Petri dish and filled with 0.1 ml of RJ or 1% chlorhexidine (CHX) gel. The plates were incubated for 24 h at 36°C. The antibacterial activity was evaluated after 24 h cultivation by measuring the diameter of the transparent areas around the wells. No transparent area was interpreted as no bactericidal effect of the test substance on the bacteria strain.

Determination of 10-HDA in various solvents by HPLC

The 10-HDA content in the samples of Lithuanian RJ were analysed by the HPLC method using the Waters 2695 chromatographic system, with a diode matrix detector Waters 996. The chromatographic conditions were the following: two eluents trifluoroacetic acid, as well as acetonitrile. The column used was ACE C18 (250 × 4.6 mm), the volume of injection 10 µl, the speed of injection 1 ml/min, temperature in the column 25°C and the wavelength for detection 210 nm. Then the concentrations were determined and summarized using the Empower 3 chromatographic Software (Waters corporation, Milford, USA).

Statistical analysis

The results are presented as means ± SD. Statistical analysis was conducted with one-way analysis of variance, followed by Dunnett's post-test with

the software package Sigma Plot 12.0 version (Systat Software Inc.). $p < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

Quality assessment

Lithuanian RJ is a slightly yellow, thick and milky viscous liquid. Usually Royal jelly is not homogeneous due to the various size undissolved granules appearing in the final product. We have evaluated several criteria for quality assessment of fresh Lithuanian RJ, such as pH, water content, quantity of polyphenolic compounds, as well as the amount of 10-HDA in various solvents, as this fatty acid is found only in Royal jelly, and it is one of the main quality markers for RJ.

The results of the quality assessment of various samples of Lithuanian RJ are presented in Table 1. The reported water content of fresh RJ is about two-thirds [17]. In agreement with those reports, we found the water content in Lithuanian RJ to be 61.1–63.9% [17–20]. Thus, the major component of RJ is water [21–23]. The pH of our samples of Lithuanian RJ was 3.7–4.1, which conforms with the reported high acidity of RJ [24, 25]. 10-HDA is one of the most important fatty acids in RJ, and because of its wide range of biological effects, it may be used as a marker for determining the quality of RJ [26–28]. We also found that the concentration of polyphenol compounds among the samples was 28–43 µg/ml.

Antiradical activity

The quantity of free radicals bound by RJ was determined with the free-radical binding 2,2-diphenyl-1-picrylhydrazyl (DPPH• assay) method [18]. Figure 1 illustrates the relationship between polyphenolic compounds and antioxidant activity in the RJ samples. The samples N1, N2 and

Table 1. Quality assessment of RJ produced in Lithuania (mean ± SD, $n = 3$)

Sample	pH	Water content, %	10-had, %	Polyphenolic compounds, µg/mg
N1	3.8 ± 0.03	61.4 ± 0.23	2.42 ± 0.05	43 ± 0.73
N2	3.7 ± 0.02	61.1 ± 0.34	2.88 ± 0.03	38 ± 0.52
N3	3.9 ± 0.02	63.7 ± 0.18	2.20 ± 0.08	40 ± 0.61
N4	4.1 ± 0.04	62.9 ± 0.51	2.08 ± 0.04	28 ± 0.32
N5	3.7 ± 0.01	62.6 ± 0.33	2.22 ± 0.10	33 ± 0.56
N6	3.8 ± 0.03	63.9 ± 0.26	2.11 ± 0.07	29 ± 0.44

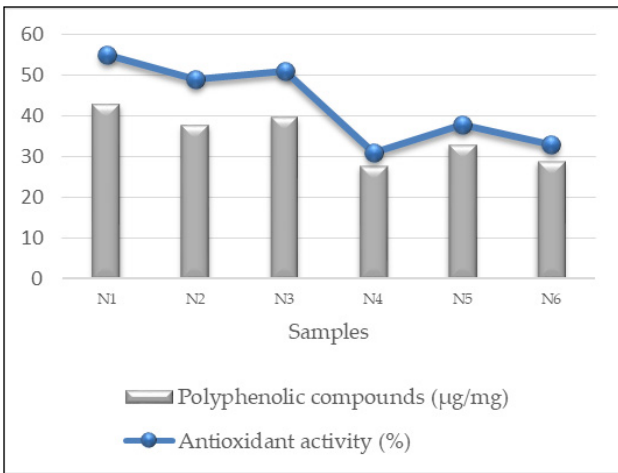


Fig. 1. Antiradical activity of RJ

N3 harvested in 2016 had more antiradical effect than did the samples N4, N5 and N6 harvested in 2017. There was a strong direct correlation between antioxidant activity and the quantity of polyphenol compounds ($R = 0.9956$). This relationship is consistent with the evidence that polyphenol compounds are the main components of RJ that determine its antioxidant activity [30]. Use of natural antioxidants may increase the stability of food

products, nutrients and medications, and bolster the anti-inflammatory, anti-allergic and anti-tumour abilities of the organism by using the polyphenol properties of the antioxidants [31]. Natural antioxidants in plants are usually obtained from polyphenols and flavonoids [32]. Our results suggest that RJ can be used as a source of antioxidants. Moreover, the quantity of phenolic compounds and the antioxidant activity are suitable criteria for the assessment of RJ [18].

Antibacterial activity

The antibacterial activity of RJ against the reference American Type Culture Collection (ATCC) strains and clinical strains is illustrated in Fig. 2. RJ suppressed best the growth of the clinical *E. faecalis* strain. The reference *E. faecalis* strain was much more resistant to the effect of RJ than was the clinical strain: RJ had 37.16% ($p < 0.05$) less effect on the reference strain than on the clinical strain. RJ had less effect on other strains of gram-positive bacteria. RJ had an approximately 30% less effect on the reference strain of *S. aureus* than on the clinical strain. RJ inhibited the growth of *S. aureus* significantly less ($p < 0.05$) than did the control. Bogdanov [15]

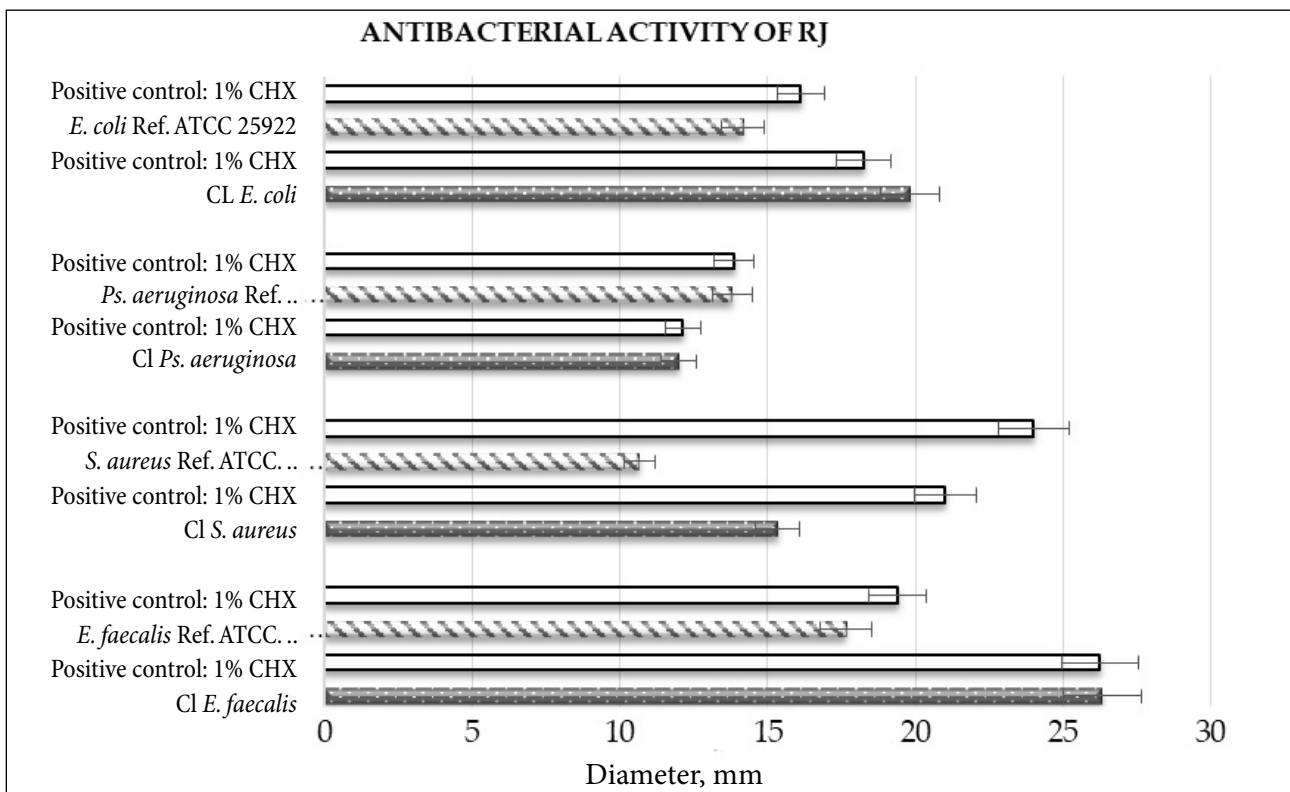


Fig. 2. Antibacterial activity of RJ. One percent chlorhexidine (CHX) gel was the positive control (mean ± SD, n = 3). Cl., clinical strains

also identified that RJ inhibits the growth of *S. aureus*. Bulgarian scientists studied the effect of honey products, including RJ, on the growth of *S. aureus* [33]. Other studies performed in the past suggested that bee products may be used as therapeutic substances due to their biological activities [34]. All bee products, including RJ, may be alternative therapeutic substances against methicillin-resistance *Staphylococcus aureus* infections, but clinical testing is needed to confirm this [34]. The analysed samples of Lithuanian RJ had activity against the gram-negative bacterium, *P. aeruginosa*. The data presented in Fig. 3 show that the clinical strain of *P. aeruginosa* was more resistant to the effect of RJ than was the reference strain. RJ inhibited the growth of *P. aeruginosa* significantly ($p < 0.05$) less than did the control sample. All analysed samples of RJ inhibited the growth of the clinical strain of *E. coli* better than they inhibited the reference strain. Studies established that RJ possesses antibacterial activity against strains of gram-positive bacteria [9]. Various studies developed the knowledge that the antibacterial activity of RJ depends on biologically active compounds in the composition, such as 10-HDA. Our research results suggest that RJ is suitable for the production of preparations with antibacterial properties [35].

Quantitative determination of trans-10-hydroxy-2-decenoic Acid (10-HDA) in various solvents

Royal jelly is a suitable candidate in medicinal and dermatological use, for its antibacterial, protective

and antioxidant effects [22, 25, 26, 29]. Solubility is one of the most important criteria in order to obtain pharmaceutical and medical formulations suitable for using both internally and externally avoiding the use of harmful and toxic solvents. 10-HDA is a fatty acid, which is best soluble in organic solvents, as ethanol, DMSO and dimethyl formamide (DMF) [26]. The solubility of the main compound in formulations depends on its structure and solution conditions, the structure determines the lipophilicity, while 10-HDA is a fatty acid and the structure is highly lipophilic [26]. Also, solution conditions are affected by pH, as well as co-solvents. The increase of solubility can dramatically enhance bioavailability [39]. The increase of pH using the phosphate buffered saline solution is important to use in ophthalmology as well as in formulations used internally. The sample chromatogram of 10-HDA in the Royal jelly phosphate buffer saline solution is illustrated below. The retention time with our validated method was 13.513 ± 0.3 min. Since 10-HDA from Royal jelly exhibits various health promoting properties, such as antibacterial, anti-tumor, etc. [26, 36], it is important to use non-toxic solvents, due to the fact that ethanol, DMSO and DMF as well as other harsh solvents commonly used in the field of production are highly toxic when ingested or used topically on sensitive areas. We have evaluated the impact of various solvents for the amount of 10-HDA in the solution, in order to achieve the quantity of the acid in aqueous solutions which are non-toxic and can be used internally

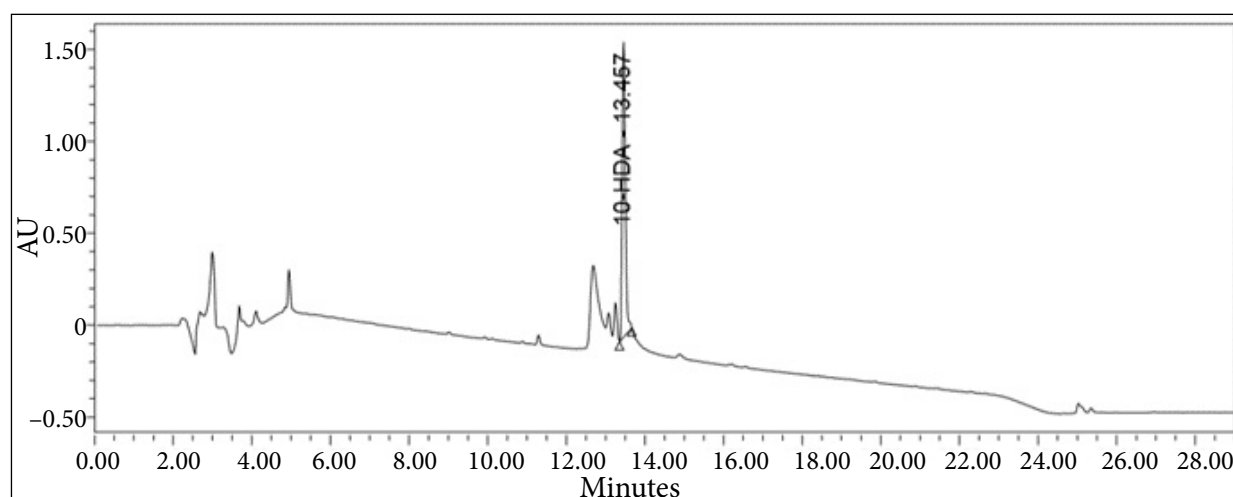


Fig. 3. A sample chromatogram of the RJ solution with phosphate buffer solution

as well as topically. Also, as a non-toxic and commonly used additive, β -cyclodextrin is a suitable co-solvent to increase the solubility in water for 10-HDA[39]. Various solvents and their impact for pH of the solutions is indicated in Table 2. It shows that addition of PBS (phosphate buffered saline) increases the pH of solutions and makes it more isotonic so it could be used more widely in medical and pharmaceutical research. As seen in Table 2, the pH of formulations varied from 3.9 up to 6.95 in the PBS solution. Phosphate buffered

saline is commonly used as an additive to obtain desirable pH values in diverse formulations, and is a non-toxic aqueous solvent, with potential use in even ophthalmologic formulations.

The quantitative determination of 10-HDA in various solvents, indicated in Table 2, was measured using the validated HPLC method. As seen in Fig. 4, the smallest amount of 10-HDA was found in physiological 0.9% NaCl solution, $65.39 \pm 2.53 \mu\text{g/ml}$. In the purified water and phosphate buffer solution the amount of 10-HDA was not significantly different ($p > 0.05$), 146.18 ± 4.65 and $141.23 \pm 3.76 \mu\text{g/ml}$, respectively. In all concentrations of ethanol the amount of 10-HDA was around 1.7 times higher than in aqueous solutions, in 30, 50 and 70% ethanol the amounts of 10-HDA were 248.25 ± 4.89 , 252.35 ± 6.53 and $276.28 \pm 5.54 \mu\text{g/ml}$, respectively. To enhance the solubility of 10-HDA in aqueous solutions, we used cyclodextrin, which is proven to enhance solubility of compounds that are less soluble in water [41]. The addition of cyclodextrins to the aqueous solution increased the amount of 10-HDA in the samples by up to the amount that was in ethanol solutions, 0.5% β -cyclodextrin with water had $253.16 \pm 7.65 \mu\text{g/ml}$ of 10-HDA, and 2% β -cyclodextrin with water had $278.26 \pm 7.34 \mu\text{g/ml}$ of 10-HDA. The amounts of 10-HDA in various ethanolic solutions and aqueous solutions with β -cyclodextrin were not significantly different ($p > 0.05$).

Table 2. Different solvents used to solubilize 10-HDA and their pH values

Solution	Solvent	pH
N1	Purified water	3.9 ± 0.03
N2	0.9% NaCl	5.2 ± 0.04
N3	Phosphate buffer (PBS)	6.95 ± 0.04
N4	30% ethanol	4.74 ± 0.03
N5	50% ethanol	4.82 ± 0.02
N6	70% ethanol	4.89 ± 0.03
N7	Purified water with 0.5% β -cyclodextrin	4.13 ± 0.04
N8	Purified water with 2% β -cyclodextrin	3.95 ± 0.02
N9	PBS with 0.5% β -cyclodextrin	6.87 ± 0.04
N10	PBS with 2% β -cyclodextrin	6.92 ± 0.03

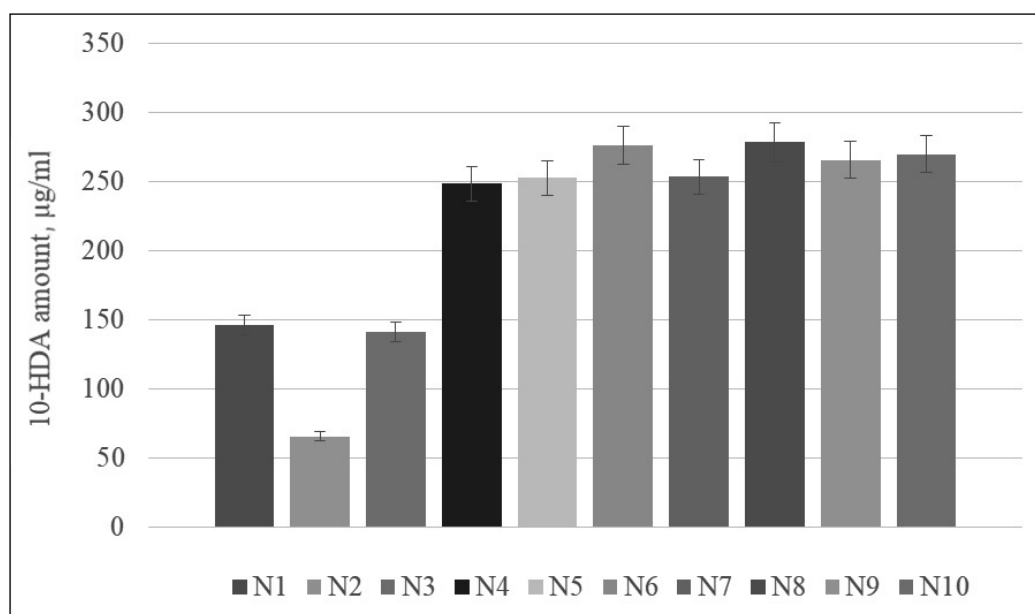


Fig. 4. Quantities of 10-HDA using various solvents, obtained by HPLC method

CONCLUSIONS

In the qualitative analysis of Lithuanian Royal jelly, the results have supported the data published in other studies that the main component in fresh Royal jelly is water, and pH of diverse samples was acid, as it was expected. The antioxidant activity of Royal jelly measured with the DPPH• assay method was directly dependent on the amount of active substances as polyphenolic compounds, as well as additional complex substances. The antibacterial analysis has shown that Lithuanian Royal jelly is effective against reference and clinical strains of the bacteria types tested. The amounts of 10-HDA, main Royal jelly fatty acid and marker for RJ quality were highest in ethanol solutions of various concentrations, as well as aqueous solutions with β -cyclodextrin addition. The results of this original study are significant for standardizing the quality assessment of Lithuanian Royal jelly, as well as its biological properties demonstrated a high potential in medicinal use.

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**LIETUVIŠKO BIČIŲ PIENELIO KOKYBĖS IR
BIOLOGINIO AKTYVUMO TYRIMAI BEI
TRANS-10-HIDROKSI-2-DECENO RŪGŠTIES
KIEKIO NUSTATYMAS NAUDOJANT ĮVAIRIUS
TIRPIKLIUS**

Santrauka

Bičių pienelis – tai bičių darbininkių išskiriamas produktas, vis labiau populiarėjantis dėl biologinių savybių ir unikalios cheminės sudėties. Tyrimo metu buvo vertinami lietuviško bičių pienelio kokybės rodikliai: vandens kiekis, antioksidacinis aktyvumas, fenolinių junginių kiekis ir pH. Rezultatai parodė, kad lietuviško bičių pienelio pH, vandens ir fenolinių junginių kiekiai sutapo su anksčiau skelbtais mokslinių tyrimų duomenimis. Įvertinta skirtingų tirpiklių įtaka 10-hidroksi-2-deceno rūgšties (10-HDA) – pagrindinės bičių pienelio sudėtyje esančios riebalų rūgšties ir bičių pienelio kokybės rodiklio – ištirpinimui taikant efektyviosios skysčių chromatografijos metodikas. Rezultatai parodė, kad 10-HDA geriausiai ištirpsta skirtingų koncentracijų etanolyje, tačiau tikslinga naudoti mažiau toksiškus tirpiklius. Į tirpalus pridėjus β -ciklodekstrino, ištirpusios 10-HDA kiekis vandeniniuose tirpaluose buvo toks pat, kaip ir etanolio tirpaluose. Atlikti antioksidacinio ir antibakterinio aktyvumo tyrimai. Jie atskleidė, kad bičių pienelis pasižymi teigiamu poveikiu prieš laisvuosius radikalus, o teigiamas antibakterinis poveikis buvo nustatytas prieš dažniausiai pasitaikančias bakterijas, pavyzdžiui, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* ir *Staphylococcus aureus*. Tyrimo rezultatai rodo, kad parinkti metodai yra reikšmingi siekiant tolimesnių tyrimų standartizavimo ir praktinio jų pritaikymo nagrinėjant bičių produktų cheminę sudėtį ir biologinį aktyvumą.