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THE PHENOLIC PROFILE OF RAPESEED HONEY ENRICHED WITH *MELILOTUS ALBUS* FLOWERS AND ITS INHIBITORY EFFECT ON RAT PLATELET AGGREGATION

S u m m a r y

Background. The *Melilotus* plants are highly valued in herbal medicine, recommended for the prevention and treatment of thrombotic vein inflammation and varicose veins. Their health-promoting properties primarily result from the presence of coumarin and its derivatives. The aim of the study was to determine whether honey enriched with dried *Melilotus albus* flowers has the ability to inhibit platelet aggregation. The inhibitory activity of rapeseed honey (control sample), rapeseed honey enriched with 1 % w/w of *M. albus* dried flowers and pure coumarin were evaluated against ADP (5 μM) and collagen (2 μg/ml) induced aggregation of rat platelets. Platelet aggregation was measured by the turbidimetric method. Moreover, the quantitative polyphenolic profile determined using HPLC-PDA and volatile compounds profiles obtained by GC-MS methods for rapeseed and *M. albus* enriched honey were compared to identify the chemical factors that limit platelet aggregation.

Results and conclusions. A beneficial, dose-dependent effect of enriched honey on the inhibition of the aggregation process was demonstrated, and for the highest concentration used (20 %), the inhibition of aggregation induced by ADP and collagen amounted to 75 % and 90%, respectively. A weaker inhibitory effect was found for pure rapeseed honey at 20 % concentration – 40 % and 55 % of inhibition, respectively and pure coumarin (0.5 mg/ml) – 44 % and 20 %, respectively. The results obtained demonstrated that rapeseed honey enriched with *M. albus* flowers possesses antiplatelet activity resulting from the synergis-

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tic effect of coumarin and other bioactive compounds occurred in dried *M. albus* flowers, including phenolic and volatile components.. However, the mechanism of this action needs further study.

Keywords: platelet aggregation, ADP, collagen, *Melilotus albus*, honey

Introduction

Malfunction of the platelet aggregation mechanism can cause the progression of thrombus formation inside the vascular channels of the body, resulting in atherosclerosis and cardiovascular diseases (CVDs). Therefore, natural substances that have the ability to inhibit platelet aggregation are sought. Among such substances, varietal honey rich in biologically active compounds, which have been found to be effective in the prevention of chronic diseases, including atherosclerosis and cardiovascular diseases, deserves special attention [3, 32].

Honey is a natural and best-known bee product, used in the treatment and prevention of diseases for centuries. Several studies demonstrated that honey consumption has beneficial effects on cardiovascular disease indicators [1, 16, 19]. The reduction in platelet activity was proved by Ahmed et al. [2], who studied the effects of different types of honey on platelet aggregation and coagulation. Recently, in order to increase the biological activity of honey, plant additives have been introduced to it. Research showed that different plant additives to honey create new health-promoting properties of the final product, depending on the pharmacological properties of the plant [12, 37]. This creates new opportunities for the production of medicinal preparations that could be an alternative to herbal medicines (tablets, tinctures), more convenient to use for the elderly. It is assumed that such products have an enhanced health-promoting effect due to the synergistic effect of herbs and honey. However, such products require research, especially clinical trials. In our previous study, we enriched multifloral honey with fresh and dried flowers of *Melilotus officinalis* and *Melilotus albus* [34]. The study showed that the addition of the *Melilotus* flowers enriched honey mostly in coumarin – a characteristic plant metabolite belonging to this species.

In herbal medicine, sweet clover (*Melilotus*) is used to treat digestive disorders, conjunctivitis, arthritis, bronchitis, haemorrhoids, stomach ulcers, swelling of the lower limbs caused by inadequate blood circulation, and externally on wounds, burns, and boils [6, 16]. It is commonly accepted that coumarin is largely responsible for the health-promoting properties of this species. Some scientific sources state that this chemical compound has the ability to inhibit the formation of blood clots [16, 20]. However, other scientific sources provide information that it is not coumarin itself that has anticoagulant properties, but its derivatives such as dicoumarol [10, 21].

Based on the information provided above, this study aimed to investigate the inhibitory effect of rapeseed honey enriched with *Melilotus albus* flowers against ADP-

and collagen-induced platelet aggregation, in comparison to the single action of coumarin and rapeseed honey used as controls. Moreover, the content of bioactive compounds was analyzed using HPLC-PDA and GC-MS methods to identify the component that may be responsible for the antiplatelet aggregation.

Materials and methods

Materials

Rapeseed honey was obtained from an ecological apiary located in the Podkarpackie region, which is in the southeastern part of Poland (49º57'09" N 22º35'31"E). *Melilotus albus* flowers were collected from the ecological crops also located in the Podkarpackie region (49°57′07″N 22°09′37″E) in July. The flowers were dried at room temperature (about 20 ± 2 °C) in laboratory conditions with good ventilation and without exposure to light. Subsequently, the dry plant materials were ground using a laboratory mill (A11 IKA, Königswinter, Germany).

Preparation of enriched honeys

The solid honey was decrystallized in a thermostatic water bath for 1.5 hours at 45 °C. A 189 g portion of liquefied honey was weighed into clean glass jars. Then, the sample was inoculated with 1 g of crystallized honey to initiate crystallization, and dried, powdered flowers were added at a concentration of 1 % w/w. The honey was creamed using a hand mixer (Zelmer ZHM1204L, Poland, 400 W). The sample was stirred for 5 min twice a day for three days. The control sample (rapeseed honey) was prepared in the same way but without additives. After the creaming process, the honey samples were stored at room temperature (20 ± 2 °C), protected from light until analysis.

HPLC analysis

Phenolic compounds were isolated using the solid-phase extraction (SPE) technique with Sep-Pak C18 cartridges (Waters, Ireland). A full description of the honey sample preparation was presented in our previous publication, Sowa et al. [34]. The identification of the analyzed compounds was carried out using a high-performance liquid chromatograph with a photodiode array detector (PDA) SYKAM S600 (Ersing, Germany). The analysis conditions included a Bionacom Velocity STR C18 column (3.0 × 100 mm; 2.5 μm) thermostated at 40 °C, an injection volume of 20 μl, a flow rate of 0.5 ml/min, and detection at λ = 280 nm (e.g. coumarin, hydroxybenzoic acids), 320 nm (e.g. hydroxycinnamons), and 360 nm (mainly flavonoids). The wavelength was selected based on the maximum absorption of each compound according to literature data. The mobile phase consisted of 5 mM ammonium acetate, 0.2 % (v/v) acetic acid in water (phase A) and acetonitrile/methanol (1:2 v/v) (phase B). Gradient elution

was used during the analysis: 70 % A (2 min), 35 % A (13 min), and then back to 70 % A (5 min).

The quantification of individual phenolic compounds was carried out using an external standard method. Five-step calibration curves were prepared for each standard calibration curve in the range of 0.005 - 0.1 mg/ml ($r^2 \geq 0.9989$). The content of o-coumaric acid glycoside and quercetin glycoside were expressed as o-coumaric acid and quercetin equivalents, respectively. Analytical standards for chromatography were purchased from Sigma Aldrich (St. Louis, MO, USA).

GC- MS analysis

Volatile compounds were extracted by headspace-solid phase microextraction (HS-SPME) using a 100 μm polydimethylsiloxane (PDMS) fiber (Supelco Ltd., Bellefonte, PA, USA). Prior to the analyses, the fiber was conditioned in accordance with the manufacturer's instructions, at 250 °C for a duration of 30 min in the injector of a gas chromatograph. A 3 g portion of honey was weighed and placed in a 10 cm^3 glass vial sealed with a screw cap equipped with a silicone septum. The exposure time of the fiber to the headspace of the honey samples was 30 min at 50 °C. Subsequently, the fiber was transferred to the injector of the gas chromatograph (temperature 250 °C), where the analytes were thermally desorbed for a duration of 5 min. The whole procedure was carried out in triplicate for each sample.

The composition of the volatile compounds was determined using a gas chromatograph Varian 450 coupled to a mass detector Varian 240 (Varian, Palo Alto, California, USA). The compounds analyzed were separated using a capillary column (30 m × 0.25 mm) with a medium-polar stationary phase of HP-5 (methylphenylpolysiloxane) and a coating thickness of 0.25 μm (Agilent J&W GC column). Helium, at a constant flow rate of 1 cm^3/min, was used as the carrier gas. The oven temperature was initially held at 50 °C for 5 min, then increased to 300 °C at a rate of 5 °C/min for 25 min, and finally held isothermally at 300 °C for 5 min. The components were identified by comparing their mass spectra with those in the spectrometer database using the NIST.08 and the Willey database with probabilities higher than 80 %. The results were expressed as a percentage of the total peak area. Additionally, for coumarin, a quantitative analysis was carried out based on a standard curve (standard addition method). The coumarin solutions (0.005 ÷ 0.1 mg coumarin/1 g honey) were introduced to the coumarin-free honey samples. A linear detector response was obtained ($r^2 = 0.9986$).

Experimental Animals

For the *in vitro* experiment, three-month-old male Wistar rats (150 to 220 g) were used. The animals were kept in clean and dry polypropylene cages under a controlled

temperature of 25 ± 2 °C, a relative humidity of 45 ÷ 55%, and a 12-hour light-dark cycle in the animal house of Ivan Franko National University of Lviv. The rats were fed *ad libitum* using a standard laboratory diet and provided with water. Before starting experimental manipulation, the animals were acclimatized for at least 7 days to adapt to the environment. The overall health of the rats was monitored every other day, and no adverse events were recorded during the housing period. The protocol used in the study was developed in accordance with the guidelines "General Principles of Work on Animals," approved by the 1st National Congress of Bioethics (Kyiv, Ukraine, 2001), and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes [7]. Compliance with the ethical principles of work was certified by the Ethics Committee of Ivan Franko National University of Lviv, Ukraine (protocol No. 23-07-2021 from 19 July 2021). The animals were sacrificed by decapitation under diethyl ether anesthesia after a 15-hour fast. Complete blood was collected and immediately transferred into tubes. Sodium citrate was used as an anticoagulant during blood collection at a 1:10 ratio.

Platelets aggregation assay

Platelet-rich plasma was obtained by centrifugation at 180 × g for 15 min at room temperature. Platelets were isolated from plasma by centrifugation at 800 × g for 15 min and resuspended in the final buffer consisting of 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.0 mM NaH_2PO_4, 5 mM glucose, 10 mM Hepes (pH 7.4), 0.2% BSA, and 1 U/cm^3 apyrase. The platelet count was adjusted to 2×10^5 cells per µl [23]. The measurements were made in the platelet suspension at 37 °C while stirring at 800 rpm for 800 sec. Adenosine diphosphate (ADP) (5 µM) and collagen (2 µg/ml) were used as aggregation agonists.

Various concentrations (1 %, 5 %, 10 % and 20 % w/v) of honey enriched with *Melilotus flowers*, and 10 µl of the solution were added following the addition of the aggregation agent. As a control, pure honey (in the same concentrations) and 10 µl of coumarin (0.5 mg/cm^3) dissolved in 0.9 % sodium chloride were used. The aggregation ability of platelets was measured by the turbidimetric method using "LA 230", a two-channel laser aggregation analyzer (SPF "Biola", Russia). The degree of aggregation was defined as the maximum light transmission (%), and the aggregate size was defined as the maximum aggregate size (c.u.). Results were presented as a percentage of aggregation inhibition (maximum light transmission in aggregation induced by ADP or collagen was taken as 100 %).

Statistical analysis

All analyses were performed in triplicate. Results are presented as mean ± standard deviation (SD). Statistically significant differences ($p < 0.05$) between rapeseed

and enriched honey based on the content of individual phenolic compounds were determined by Student's t-Test for Independent Samples, whereas the aggregation ability of platelets of the honey samples in comparison to pure coumarin was checked using one-way analysis of variance (ANOVA) with Dunnett's test ($p < 0.05$).

Results and discussion

Rapeseed honey is classified as pale honey and is characterized by a low content of bioactive compounds, including phenolic compounds [8]. In addition, rapeseed honey crystallizes very quickly, which makes it a very good variety that can be used for enrichment with plant additives [37]. In general, the phenolic compounds found in honey primarily belong to two groups: phenolic acids and flavonoids. The rapeseed honey studied contained several phenolic compounds only, and a higher content of phenolic acids compared to flavonoids was found, in particular, hydroxybenzoic acid derivatives, such as p-hydroxybenzoic acid, vanillic acid and syringic acid (Table 1). Surprisingly, we did not identify quercetin in raw rapeseed honey, which is one of the most common compounds in food and natural honeys and was identified in other studies [5, 25, 33]. It is commonly accepted that the phenolic profile of honey samples is greatly influenced by geographical location and floral sources; therefore, there are significant differences in the concentrations and profiles of these compounds in individual varietal honeys [36]. However, large variation in the content of individual phenolic compounds within the same variety, including rapeseed honey, was found in research Wen et al. [39]. Similarly to our results, the p-hydroxybenzoic, caffeic, ferulic and syringic acid content in rapeseed honey was confirmed by Kędzierska-Matysek et al. [25], while apigenin, kaempferol and chrysin, among flavonoids, were also detected.

The addition of *Melilotus albus* flowers enriched the rapeseed honey studied with seven phenolic compounds that were not identified in pure nectar honey (Table 1). Importantly, no statistically significant differences were found in the content of other phenolic compounds ($p > 0.05$). The enrichment resulted in over a tenfold increase in the total content of phenolic compounds.

The compound whose content was found to be the highest was coumarin (73.12 mg/kg), a characteristic metabolite of plants of the genus *Melilotus.* Furthermore, high enrichment in o-coumaric acid, hyperoside and quercetin was observed. These compounds were identified in the flowers of *Melilotus albus* (unpublished results). High enrichment in coumarin in multifloral honey combined with *Melilotus flowers* was observed in our previous preliminary research [34], however, at that time, we did not analyze other bioactive compounds. The additive modifies the composition of the phenolic compounds of the final product, as proven by other scientists who added flowers and leaves of *Morus alba* [37], *Spirulina platensis* [13], propolis [14] and bee pollen [15].

Table 1. The content of individual phenolic compounds in rapeseed honey (control) and honey enriched with 1 % (w/w) of dried *Melilotus albus* flowers

Tabela 1. Zawartość poszczególnych związków fenolowych w miodzie rzepakowym (kontrola) oraz miodzie wzbogaconym 1% (m/m) dodatkiem suszonych kwiatów *Melilotus albus*

Compound / Związek	Retention Time / Czas retencji (min)	Rapeseed honey / Miód rzepakowy (mg/kg)	Honey enriched with 1 % *M. albus* flowers / Miód wzbogacony 1 % dodatkiem kwiatów *M. albus* (mg/kg)
o-coumaric acid glycoside / glikozyd kwasu o-kumarowego	3.03	<LOD	2.07 ± 0.01
p-hydroxybenzoic acid / kwas p-hydroksybenzoesowy	3.34	1.63 ± 0.04[a]	1.62 ± 0.03[a]
quercetin glycoside / glikozyd kwercetyny	3.65	<LOD	4.45 ± 0.02
vanillic acid / kwas wanilinowy	3.81	1.61 ± 0.04[a]	1.61 ± 0.02[a]
caffeic acid / kwas kawowy	4.38	1.41 ± 0.03[a]	1.41 ± 0.05[a]
ferulic acid / kwas ferulowy	5.42	0.12 ± 0.01[a]	0.11 ± 0.01[a]
syringic acid / kwas syryngowy	6.21	5.82 ± 0.03[a]	5.63 ± 0.04[a]
coumarin / kumaryna	6.40	<LOD	73.12 ± 0.05
o-coumaric acid / kwas o-kumarowy	6.92	<LOD	6.26 ± 0.01
hyperoside / hiperozyd	7.17	<LOD	21.11 ± 0.01
sinapic acid / kwas synapinowy	7.27	0.25 ± 0.07[a]	0.26 ± 0.04[a]
hesperetin / hesperetyna	9.80	0.09 ± 0.01[a]	0.09 ± 0.02[a]
naringenin / naringenina	11.18	0.29 ± 0.14[a]	0.33 ± 0.06[a]
luteolin / luteolina	13.90	<LOD	0.80 ± 0.01
quercetin / kwercetyna	14.70	<LOD	7.71 ± 0.00
apigenin / apigenina	15.38	0.73 ± 0.01[a]	0.75 ± 0.02[a]
kaempferol / kemferol	15.59	0.26 ± 0.00[a]	0.25 ± 0.01[a]
chrysin/ chryzyna	17.33	0.14 ± 0.01[a]	0.16 ± 0.03[a]
Total/ Suma		12.35 ± 0.40	127.74 ± 0.11

Explanatory notes / Objaśnienia:
LOD – the limit of detection/ LOD – poniżej granicy wykrywalności;
[a]Mean values ± standard deviation with different superscripts in the same row are significantly different ($p < 0.05$) / [a]Wartość średnia ± odchylenie standardowe, różne litery w tym samym wierszu oznaczają różnice statystycznie istotne ($p < 0.05$)

Due to the fact that coumarin is a volatile compound and the technique of GC-MS is more commonly used for its determination, we conducted a comparative analysis using this technique. The content of coumarin determined by the GC-MS method was 62.72 mg/kg, which is similar to that obtained by the HPLC-PDA method (73.12 mg/kg). The difference observed most likely results from various sample preparation methods. In the case of HPLC-DAD, the SPE method was employed, while in GC-MS, the SPME method was used. What is extremely important, this compound was not identified in the rapeseed honey (control sample). Moreover, the enrichment

strongly modified the profile of volatile compounds (Table 2). The addition resulted in a significant abundance of coumarin dominating the volatile compound profile. Apart from coumarin, nonanoic acid; eugenol; 2,6-diethyl-1,4-benzoquinone; and 3,4-dihydro-2H-1-benzopyran-2-one (also known as 3,4-dihydrocoumarin) were identified in the enriched honey, but at much lower levels. This means that the potential health-promoting properties, as well as the taste and aroma of the honey enriched with *M. albus* flowers, will be mainly shaped by coumarin. What is of particular importance is the fact that, while searching scientific databases, we did not find publications where the effect of honey enrichment on the profile of volatile compounds was analyzed.

Table 2. Effect of addition of *Melilotus albus* flowers (1 % w/w) on the profile of volatile compounds of rapeseed honey identified by GC-MS

Tabela 2. Wpływ dodatku kwiatów *Melilotus albus* (1 % w/w) na profil związków lotnych miodu rzepakowego wyznaczony za pomocą GC-MS

Compound / Związek	Rapeseed honey / Miód rzepakowy	Honey enriched with 1 % (w/w) *M. albus* flowers / Miód wzbogacony 1 % (m/m) dodatkiem kwiatów *M. albus* (mg/kg)
	Peak Area/ Powierzchnia piku (%)	
phenylethyl alcohol/ alkohol fenyloetylowy	6.09	0.66
4,4-dimethyl-2-propenylcyclopentanone/ 4,4-dimetylo-2-propenylocyklopentanon	3.51	0.14
lilac aldehyde/ aldehyd liliowy	1.19	0.12
heptanediamide, N,N'-di-benzoyloxy-/ N, N'-di-benzoiloksy-heptanoamid	5.88	0.13
phenol, 2-methyl-5-(1-methylethyl)-/ 2-metylo-5-(1-metyloetylo)-fenol	10.19	0.21
nonanoic acid/ kwas nonanowy	< LOD	0.13
eugenol/ eugenol	< LOD	0.26
3,5-dimethoxybenzaldehyde/ 3,5-dimetoksybenzaldehyd	3.72	0.14
2H-1-benzopyran-2-one (coumarin)/ 2H-1-benzopiran-2-on (kumaryna)	< LOD	95.80
3,4-dihydro-2H-1-benzopyran-2-one / 3,4-dihydrokumaryna	< LOD	0.40
succinic acid, di(tridec-2-ynyl) ester/ bis (tridec-2-ynylo) butanodionian	3.48	0.14
propanoic acid, 2-methyl-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester/ ester 2-metylo-1,1-dimetyloetylo-2-metylo-1,3-propanodiylowy kwasu propanowego	23.94	0.16
1H-indene, 2-butyl-5-hexyloctahydro/ 2-butylo-5-heksylo-oktahydro-1H-inden	41.28	1.60
2,6-diethyl-1,4-benzoquinone/ 2,6-bis (1,1-dimetyloetylo)- 2,5-cykloheksadien-1,4-dion	< LOD	0.11

A platelet aggregation blood test checks how well platelets, a part of blood, clump together and cause blood to clot. Antiplatelet agents inhibit clot formation by preventing platelet activation and aggregation, while anticoagulants primarily inhibit the coagulation cascade and fibrin formation [38]. The aggregation ability of platelets was measured by the turbidimetric method. This method is based on the relative variance of optical density fluctuations caused by random changes in the number of units that fall in the path of the laser beam, which reflects deviations from their average size. For the formation of a plug and restoration of blood patency during vessel damage, platelets must be activated [30]. Collagen and adenosine diphosphate (ADP) are the two agonists most frequently used as platelet aggregation persuaders to estimate the effects of antiplatelet drugs. However, the latest study showed that the use of traditional agonist ADP could be insufficient and recommended combining it with collagen-induced aggregation test [35]. Therefore, we decided to compare the effect of both agonists in our study.

Adenosine diphosphate (ADP) is released from red blood cells and endothelial cells upon vessel damage. ADP belongs to the group of so-called weak platelet agonists that directly induce platelet aggregation without triggering secretion. After stimulation with this agonist, platelets undergo shape changes and produce thromboxane A2. In addition, ADP activates the fibrinogen receptor, causing platelets to bind fibrinogen and aggregate. ADP also leads to platelet aggregation by the activation of P2TAC and P2Y1 receptors [23]. It was found that both the pure rapeseed honey and the honey enriched with *Melilotus flowers* have a significant, concentration-dependent inhibitory effect against ADP-induced platelet aggregation. Honey enriched with *M. albus* flowers at a concentration of 20 % w/v possesses the greatest inhibitory ability (Fig. 1). The honeys studied inhibited, at the lowest concentrations (5 % and 1 % w/v), ADP aggregation to a lesser extent than synthetic coumarin, by between 13 % and 17 % ($p < 0.05$). It can be assumed that the biologically active substances from the honey samples studied prevent the binding of ADP to platelet receptors, thereby competitively antagonizing ADP receptor-mediated aggregation.

Collagen is classified as a strong agonist, and collagen-induced platelet activation takes place under physiological conditions. Strong agonists directly induce platelet aggregation, thromboxane A2 (TxA2) synthesis and platelet granule secretion [4]. We found that 20 % and 10 % (w/v) pure rapeseed honey has much higher potential to inhibit platelet aggregation compared to synthetic coumarin. However, at lower concentrations, this sample of honey did not have such a pronounced inhibitory ability (Fig. 2). The concentration of honey solution enriched with dried flowers had a significant impact on the inhibition of the aggregation process, the beneficial effect was already observed at a concentration of 1 % (w/v). The enriched honey showed a much higher ability to inhibit platelet aggregation compared to coumarin and rapeseed

honey solutions, which indicates that other bioactive compounds found in *M. albus* are involved in the process. Speculatively, it can be assumed that honey enriched with *M. albus* flowers is able to affect platelet granule secretion and TxA2 synthesis, however, such an assumption needs further experimental confirmation.

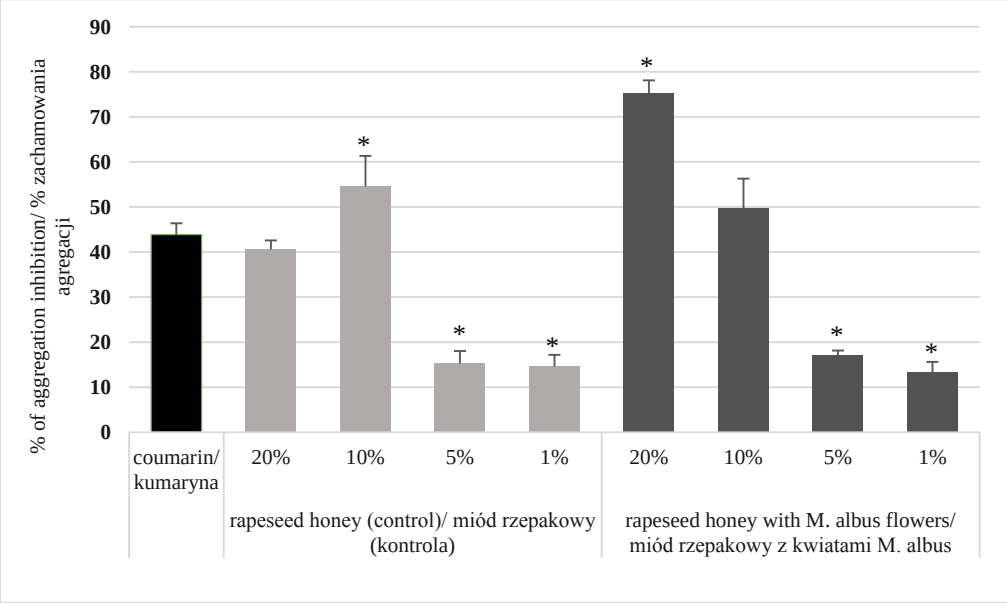

Fig. 1. Effect of coumarin, rapeseed honey and honey with 1 % (w/w) *Melilotus albus* dried flowers resulting in inhibition of ADP-induced platelet aggregation

Rys. 1. Wpływ kumaryny, miodu rzepakowego i miodu z 1 % (m/m) dodatkiem suszonych *kwiatów Melilotus albus* na zahamowanie procesu agregacji płytek krwi indukowanej przez ADP

Explanatory notes / Objaśnienia:

*statistically significant differences compared to coumarin ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Dunnett's test/ *-różnice statystycznie istotne w porównaniu do kumaryny ($p < 0,05$), wyznaczono za pomocą jednoczynnikowej analizy wariancji (ANOVA) z testem Dunnetta

In the study by Li et al. [27], the roles of collagen and ADP in assessing the effects of aspirin or clopidogrel on platelet aggregation were analyzed. They observed the differentiated actions of these two platelet agonists, i.e. ADP was better to evaluate the inhibitory effect of clopidogrel, but collagen for aspirin effects. Liu et al. [28] found that pimpinellin, a coumarin-like compound extracted from *Toddalia asiatica*, inhibited collagen-induced platelet aggregation, but did not alter ADP- and thrombin-induced aggregation. Platelet aggregation is a complex phenomenon likely involving several intracellular biochemical pathways. It is known that agonists activate platelets in a selective manner by a specific receptor, followed by a series of further signaling

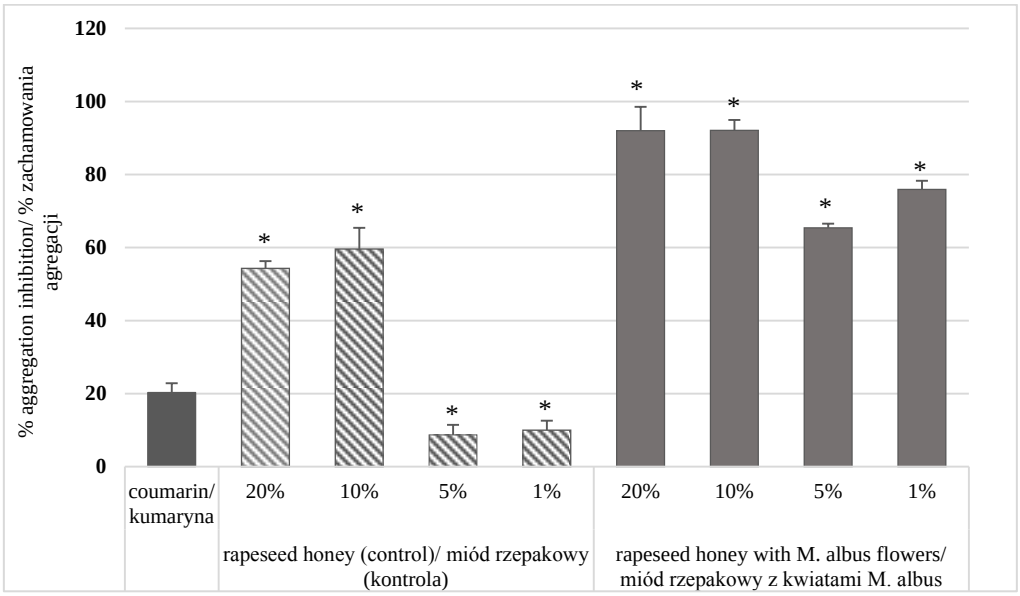

Fig. 2. Effect of coumarin, rapeseed honey and honey with 1 % (w/w) *Melilotus albus* dried flowers resulting in inhibition of collagen-induced platelet aggregation

Rys. 2. Wpływ kumaryny, miodu rzepakowego i miodu z 1 % (m/m) dodatkiem suszonych kwiatów *Melilotus albus* na zahamowanie agregacji płytek krwi indukowanej kolagenem

Explanatory notes / Objaśnienia:

* statistically significant differences compared to coumarin ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Dunnett's test/ *-różnice statystycznie istotne w porównaniu do kumaryny ($p < 0,05$), wyznaczono za pomocą jednoczynnikowej analizy wariancji (ANOVA) z testem Dunnetta

events. Collagen interacts with the immune-like glycoprotein VI receptor, which signals through an immunoreceptor tyrosine-based activation motif and activates the tyrosine phosphorylation pathway, whereas agonists like ADP interact with G protein-coupled receptors. Moreover, the aggregation mechanisms may be different [41].

Blood platelet activation and aggregation play an important physiological role in hemostasis, and it is well established that many cardiovascular disorders are linked to the abnormal and excessive activation of platelets. Thus, the inhibition of platelet hyperactivation is an important approach for treating cardiovascular disorders, and phenolic compounds are particularly attributed to the antiplatelet aggregation activity of plant extracts [9, 11, 29]. Taking this into account, it would be expected that the combination of two active ingredients: honey, a natural product obtained by bees from the nectar of flowers containing polyphenols of plant origin, and sweet clover, a plant recommended for varicose veins or poor blood circulation, would result in an enhanced synergistic effect. Bioactive compounds of plants or fruits have different mechanisms

of antiplatelet action through additive, cooperative, or synergistic effects, as presented by Fuentes et al. [11].

The research showed that the main compound identified in the product obtained was coumarin. Coumarin and its derivatives exhibit antiplatelet activity, which was proven by other researchers [22, 28, 40]. Jiménez-Orozco et al. [22] found that coumarin inhibited epinephrine and collagen-induced aggregation in a concentration-dependent manner (50, 100, 200, and 400 μM), but did not reduce ADP-induced aggregation. In the study by Zaragozá et al. [40], it was found that coumarin exhibited 25.75 % antiplatelet activity and 7.90 % occupancy of GPIIb/IIIa receptors, which was the lowest value obtained among the analyzed compounds (naringin, naringenin, esculetin and fraxetin). Additionally, they pointed out that the antiplatelet effect of coumarin was not linked with a possible interaction over blood coagulation. Such an observation was made by Kang et al. [24], who suggested that the potent antithrombotic activity of catechins (GTC) from green tea resulted from antiplatelet activity, rather than from anticoagulant activity. The strong ability of enriched honey to inhibit platelet aggregation is most likely due to the synergistic effect of several bioactive compounds, not only coumarin. Studies indicate that the antiplatelet effect is mainly attributed to the high content of flavonoids [11]. In the product tested, a high content of hyperoside was found, a compound with recognized anti-platelet aggregation activity [26]. Another compound with known antiplatelet activity is quercetin. Quercetin has been reported to inhibit collagen-stimulated platelet aggregation through the inhibition of multiple components of the glycoprotein VI signaling pathway [18], as well as inhibited platelet aggregation induced by ADP and thrombin [31]. The data quoted above confirms the hypothesis that the antithrombotic effect of honey with the addition of sweet clover flowers is the resultant effect of the synergistic action of coumarin and other polyphenolic components derived from plant additives added to honey.

Conclusions

1. The studies showed that enriching rapeseed honey with *Melilotus albus* flowers during the creaming process allows one to obtain a new product with an increased content of phenolic compounds, especially coumarin, as well as other compounds such as hyperoside and quercetin.
2. The enriched honey possessed the ability to inhibit collagen and ADP-induced aggregation in a concentration-dependent manner. The best results were observed in the case of collagen-induced aggregation for 20 % and 10 % w/v of enriched honey solution, close to 100 %. The pure rapeseed honey and coumarin solution also inhibited collagen and ADP-induced aggregation, but to a much lesser extent.

3. The results obtained indicate a synergistic effect between the bioactive compounds present in honey and white sweet clover flower itself, leading to an increase in antiplatelet activity.
4. Enriching honey with this plant additive allowed us to obtain a new product with beneficial health properties. Our study demonstrated for the first time that designed honey with sweet clover flowers may be applicable to the treatment or prevention of platelet aggregation complications linked to cardiovascular diseases. However, mechanisms of its action require further study.

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PROFIL ZWIĄZKÓW FENOLOWYCH MIODU RZEPAKOWEGO WZBOGACONEGO DODATKIEM KWIATÓW MELILOTUS ALBUS ORAZ JEGO WPŁYW NA AGREGACJĘ PŁYTEK KRWI SZCZURÓW

S t r e s z c z e n i e

Wprowadzenie. Rośliny z rodzaju *Melilotus* są bardzo cenione w ziołolecznictwie, polecane w profilaktyce i leczeniu zakrzepowego zapalenia żył oraz żylaków. Ich właściwości prozdrowotne wynikają przede wszystkim z zawartości kumaryny i jej pochodnych. Celem pracy było określenie, czy miód wzbogacony dodatkiem suszonych kwiatów *Melilotus albus* wykazuje zdolność do hamowania agregacji płytek krwi. Aktywność hamującą miodu rzepakowego (próba kontrolna), miodu rzepakowego wzbogaconego 1 % m/m dodatkiem suszonych kwiatów *M. albus* i czystej kumaryny oceniano wobec agregacji płytek krwi szczurów indukowanej ADP (5 µM) i kolagenem (2 µg/ml). Agregację płytek krwi mierzono metodą turbidymetryczną. Ponadto, ilościowy profil polifenolowy wyznaczony metodą HPLC-PDA i profil lotnych składników zapachowych określony metodą GC-MS dla miodu rzepakowego i wzbogaconego *M. albus* został porównany w celu ustalenia czynników chemicznych hamujących agregację płytek krwi.

Wyniki i wnioski. Przy najwyższym zastosowanym stężeniu (20 %) hamowanie agregacji indukowanej ADP i kolagenem wyniosło odpowiednio 75 % i 90 %. Słabszy efekt stwierdzono dla czystego 20 % miodu rzepakowego, odpowiednio – 40 % i 55 % oraz czystej kumaryny (0,5 mg/ml), odpowiednio 44 % i 20 %, wobec ADP oraz kolagenu. Uzyskane wyniki wykazały, że miód rzepakowy wzbogacony dodatkiem kwiatów *M. albus* wykazuje zdolność do hamowania agregacji płytek krwi, co wynika z synergicz-

nego działania kumaryny oraz innych biologicznie aktywnych związków występujących w suszonych kwiatach *M. albus*, w tym związków fenolowych i lotnych. Mechanizm tego działania wymaga jednak dalszych badań.

Słowa kluczowe: agregacja płytek krwi, ADP, kolagen, *Melilotus albus,* miód