NATURAL DRUGS

EVALUATION OF THE TOTAL FLAVONOID CONTENT AND ANTIMICROBIAL ACTIVITY OF THE TINCTURES OF PROPOLIS OF UKRAINIAN ORIGIN

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Abstract: Six tinctures were prepared from four different propolis samples of Ukrainian origin. The total flavonoid content (TFC) in the tinctures and propolis samples was determined by the aluminum-chloride method. The TFC of the propolis samples ranged from 4.78% to 7.18% and 11.0% to 19.5% with reference to quercetin and rutin, respectively. The TFC of the propolis tinctures was in the range of 4784.31 mg/L $\pm$ 8.80% to 6796.51 mg/L $\pm$ 0.52% and of 10411 mg/L $\pm$ 3.47% to 18499.20 mg/L $\pm$ 0.52% with reference to quercetin and rutin, respectively. The results of the TFC determination indicated a high content of flavonoids in propolis of Ukrainian origin. The best antibacterial activity of four propolis tinctures (three developed tinctures and one purchased tincture) was found against Gram-positive bacteria: *S. aureus*, *S. pyogenes* and *C. diphtheriae*. The activity of the tinctures against *E. coli* 25922 ATCC and a clinical isolate of *C. tropicalis* was absent. According to the results of this study, the tinctures of propolis of Ukrainian origin are rich in flavonoids and have good antibacterial activity against Gram-positive microorganisms. These tinctures could be regarded as preparation for the development of antimicrobial medicinal products for the complementary treatment of infectious diseases of the oral cavity.

Keywords: propolis, tinctures, flavonoids, antimicrobial activity

Such beekeeping products as honey, bee bread, and propolis have been used for a long time as food products and medical products of traditional medicine (1-6).

Propolis is a well-known resinous material collected by bees from exudates and buds of plants, mixed with pollen, bee enzymes, and wax (7). Propolis is a lipophilic material that is hard and easily broken at room temperature but elastic, soft, and very viscous during warming. It possesses an enjoyable aromatic smell and different colors, including red, green, and brown (8).

Propolis is known in traditional medicine for its numerous pharmacological activities: antibacterial, antifungal, antiviral, antioxidant, antitumor, anti-inflammatory, immunomodulatory, hepatoprotective, wound healing, cytotoxic, etc. (5, 9-14).

Over 500 substances were revealed in propolis up to 2012 (13). Propolis usually contains resins (45-55%), waxes (up to 25-35%), essential and aromatic oils (5-10%), pollen (5%), vitamins, microelements, sugars, fatty acids, flavonoids, terpenes (monoterpenes, sesquiterpenes, diterpenes, triterpenes), phenolic acids (caffeic acid, *p*-coumaric

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acid, *trans*-ferulic acid, *trans*-cinnamic acid), aminoacids, stilbenes, lignans, coumarins, *n*-alkanes, *n*-alkanes, etc. (12-19).

Among flavonoids are ones of all the classes, especially flavons (luteolin, apigenin, chrysin, and their derivatives), flavonols (macarangin, quercetin derivatives, kaempferol, galangin), flavanones (propolin A, propolin B, propolin E, 2S-naringenin, pinocembrin), chalcones, dihydrochalcones, etc. (12, 13, 15, 16, 20). Flavonoids significantly contribute to the pharmacological activities of propolis (antibacterial, antiviral, anti-inflammatory, antioxidant, immunomodulatory properties) (13, 16, 17, 19, 21). Therefore, the total flavonoid content (TFC) could be regarded as a criterion for the quality evaluation of propolis and its tinctures (13, 20). Moreover, flavonoids and aromatic acid derivatives are stated to show antimicrobial activity (13, 14, 21). Additionally, there are differences in the chemical composition depending on the country of propolis origin, the season of the propolis collection, and the proximity of herbal recourses to a hive (15, 16, 19, 21, 22). Thus, the standardization of propolis seems to be impossible for any identified or unidentified component.

The ethanolic extracts of propolis inhibited the growth of *Staphylococcus aureus*, *Enterococcus* spp., *Bacillus cereus*, *Candida yeasts* (17, 19, 23). It is supposed that the antimicrobial activity mechanism is complex and could be attributed to the synergic effect of phenolic substances, especially flavonoids and esters of phenolic acids (13, 17, 19, 21, 24).

Propolis administration reduces the use of antipyretics and anti-inflammatory medicinal products, and the rate of evolution to tracheitis, bronchitis, and rhinosinusitis at viral pharyngitis (25). The ethanolic extract of propolis (a ratio of propolis to the extract was 1 to 10, 70% ethanol as a solvent) improved the total mitochondrial respiratory efficiency in human spermatozoa *in vitro* (12). The propolis extract synergically increases the efficacy of antibiotics, including vancomycin and oxacillin against drug-resistant microorganisms (methicillin-resistant *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*) (14, 15). Propolis was stated as a healing subsidiary component for the administration of oral hygiene (14). Propolis from Pakistan has good antimicrobial activity against such pigmented anaerobic periodontal pathogens as *Porphyromonas asaccharolytica*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella melaninogenica* (26).

As a rule, for studies authors employ extracts of propolis prepared in a ratio of propolis to a final extract as 1 to 10, using 70% ethanol, heating, and maceration for 24 h (12, 15, 16).

To the best of our knowledge, there a few studies related to the determination and standardization of TFC in propolis tinctures and screening of antimicrobial activity of propolis of Ukrainian origin. Moreover, there are few studies related to the antimicrobial activity of propolis against *Corynebacterium diphtheriae* NCTC10356 and a clinical isolate of fungus *Candida tropicalis*. These stan-

Table 1. Developed tinctures of propolis of Ukrainian origin.

N	Identification of tincture	Detailed characteristics of propolis	Quantity of components	Technology	Ratio of propolis to tincture	Note
T1	10318O	Collection from 16 September 2017, unpurified, Mykolaiv region	5.01 g: 60 mL 60% ethanol	At a temperature of 40 to 50°C for 200 min plus at room temperature for 21 h	1 : 10.88	-
T2	10120S	Collection from August 2019, unpurified, Poltava region	2.70 g: 30 mL 70% ethanol		1 : 10.20	-
T3	10320S		2.50 g: 28 mL 70% ethanol		1 : 10.0	Simultaneous preparation; tincture
T4	20320O	Collection from April 2019 unpurified, Mykolaiv region	2.50 g: 28 mL 70% ethanol		1 : 10.0	10320S is more yellow
T5	40420S	Collection from August 2018, unpurified, Poltava region	5.02 g: 55 mL 70% ethanol		1 : 10.54	The same sample of propolis was used
T6	50520S		10.06 g: 111 mL 70% ethanol		1 : 9.94	

dardized tinctures could be regarded as antimicrobial, anti-inflammation, and immunomodulation preparations for the oral drug administration and oral hygiene (14, 26). Therefore, the purpose of our study was to elaborate propolis tinctures and analytical procedure for the evaluation of the developed tinctures and study their stability, and conduct screening for the antimicrobial activity of the tinctures of propolis of Ukrainian origin.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals and solvents used for analyses were of analytical purity grade. Ethanol and aluminum chloride hexahydrate were purchased from POCH S.A. (Gliwice, Poland). The rutin and quercetin were purchased from Sigma-Aldrich (Poznań, Poland).

Material

Crude propolis was collected from two regions of Ukraine in 2017-2019 (four samples). The propolis samples were kept at a temperature of 2-8°C before the tincture preparation.

Extraction

All the tinctures were obtained in a ratio of propolis to a final tincture as approximately 1 to 10. As a solvent, 70% ethanol was used. The mixtures were in closed containers at a temperature of 40-50°C for 200 min with the following extraction at room temperature for 21 h. After this period the residue was separated from the extraction solvent by means of filtration through a paper filter. Therefore, the total period of maceration was 24 h (3 h + 21 h = 24 h). The characteristics of the propolis samples and prepared tinctures are presented in Table 1.

Total flavonoid content (TFC)

TFC was evaluated by the colorimetric method as flavones, isoflavones, and flavonols form stable complexes with aluminum chloride according to C.-C. Chang et al. and Falcão et al. (5, 20). TFC was determined using the slightly modified analytical procedures of differential spectrometry provided by A. Meda et al. for the quality evaluation of the TFC in honey (27) and by N. Hudz et al. for bee bread and *Satureja montana* tinctures (2, 28). The Quercetin dihydrate and rutin trihydrate dissolution in 50% ethanol was carried out with the aid of ultrasound and temperature. The results were expressed as mg quercetin or rutin equivalents per one liter of a tincture (mg/L) or % quercetin or rutin equivalents in the samples of propolis (%).

50 µL of a stock solution of rutin trihydrate (1000 mg/L) were mixed with 0.95 mL of 50% ethanol and 1.0 mL of 2% aluminum chloride hexahydrate in 50% ethanol. After incubation at room temperature for 65 ± 5 min, the spectra of the reaction mixtures were measured in the range of 360 nm to 460 nm with spectrophotometer "Photometry Hitachi U-2810". The volume of 2% aluminum chloride hexahydrate in 50% ethanol was substituted by the same volume of 50% ethanol in the blank for rutin trihydrate. In a like manner, 50 µL of the diluted developed tinctures of propolis (1 to 20) were diluted with 50% ethanol up to 1.0 mL and was mixed with 1.0 of 2% solution of aluminum chloride hexahydrate. The mixture was mixed by vortex and incubation was done at room temperature for 65 ± 5 min. The volume of 2% solution of aluminum chloride was substituted by the same amount of 50% ethanol in the blank. The test was carried out for each tincture in triplicate.

TFC with reference to rutin was calculated using the following formula:

$$C = A_{\text{test}} \times m \times 1000 \times k / (A_R \times V_R),$$

where C is TFC of the tested tincture (mg/L), A_{test} is the absorbance of the reaction mixture of the tincture at the absorption maximum, A_R is the absorbance of the reaction mixture of rutin trihydrate solution in a concentration of 50 mg/L at the absorption maximum ($\lambda_{\text{max}} = 412$ nm), m (mg) is mass of rutin trihydrate for the preparation of the stock solution (approximately 1000 mg/L), k is coefficient for the recalculation of rutin trihydrate into rutin (0.917) and dilution of the tinctures (20 times), V_R is a volume in which rutin was dissolved. For computing with reference to quercetin, we employed the same analytical procedure, taking 50 µL of a stock solution of quercetin dihydrate (432 mg/L). TFC with reference to quercetin was calculated, using the following formula: $C = A_{\text{test}} \times m \times 1000 \times k / (A_Q \times V_Q)$, where C is TFC of the tested tincture (mg/L), A_{test} is the absorbance of the reaction mixture of the tincture at the absorption maximum, A_Q is the absorbance of the reaction mixture of quercetin dihydrate solution in a concentration of 21.6 mg/L at the absorption maximum ($\lambda_{\text{max}} = 426-427$ nm), m (mg) is mass of quercetin dihydrate for the preparation of the stock solution (approximately 432 mg/L), k is coefficient for the recalculation of quercetin dihydrate into quercetin (0.894) and dilution of the tinctures (20 times), V_Q is a volume in which quercetin dihydrate was dissolved.

Screening antimicrobial activity

For this study, we used the procedure described by Serrano et al. for herbal extracts and essential oils (29). The antimicrobial activity of three developed propolis tinctures and one tincture purchased in a pharmacy (Ukrainian manufacture “Ternopil pharmaceutical factory”, lot 30216) was evaluated against microorganisms. The tested microorganisms were Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *C. diphtheriae* NCTC 10356, clinical isolates of methicillin-resistant (MR) *S. aureus* and *S. pyogenes* (2 isolates), Gram-negative bacterium: *Escherichia coli* ATCC 25922, and fungus *C. tropicalis* isolated from a female patient. The preparation of inoculates and media was carried out in accordance with EUCAST guidelines (30, 31).

Sterile filter discs (diameter 6 mm, manufacture “Aspect”, Ukraine) were impregnated with 20, 40 and 60 μ L of four tinctures of propolis and 0.12% solution of chlorhexidine digluconate and dried in the air. Impregnating the discs with 40 and 60 μ L was conducted using 20 μ L with the following drying and putting again 20 μ L. Then dried discs were placed on the surface of Mueller-Hinton agar for *S. aureus*, *E. coli*, *S. pyogenes* and *C. diphtheria*, and Sabouraud agar (Merck, Darmstadt, Germany) for *C. tropicalis*. The treated Petri dishes with *S. aureus* and *E. coli* were incubated at a temperature of 37°C for 24 h, with *S. pyogenes* and *C. diphtheriae* for 48 h, and with *C. tropicalis* at a temperature of 30°C for 24 h.

The antimicrobial activity was evaluated by measuring the growth inhibition zone diameter, including the disc diameter. The standard discs with antimicrobial drugs (penicillin G, amikacin, ampicillin, pefloxacin, tetracycline) were used for the

quality control of the experiments. Each experiment was carried out in triplicate.

Statistical analysis

Values were expressed as means $\pm$ standard deviation for the TFC in propolis tinctures and samples and for antimicrobial activity and means $\pm$ relative standard deviation (for the TFC in propolis tinctures).

Wayne’s statistical analysis was used for comparison of the TFC mean values obtained in different days, for different samples of propolis, etc. The decision rule in all cases was: with $\alpha = 0.025$, critical values of t^* should be in the range of -2.78 to $+2.78$. Null hypothesis (H_0) was denied if $t^* < -2.78$ or $t^* > +2.78$ (2, 28, 33).

RESULTS AND DISCUSSION

The propolis samples of Ukrainian origin and respectively tinctures are rich in flavonoids. The TFC ranged from 4.78% to 7.18% and 11.0% to 19.5% with reference to quercetin and rutin, respectively. The TFC of the propolis tinctures was in the range of $4784.31 \text{ mg/L} \pm 8.80\%$ to $6796.51 \text{ mg/L} \pm 0.52\%$ and of $10411 \text{ mg/L} \pm 3.47\%$ to $18499.20 \text{ mg/L} \pm 0.52\%$ with reference to quercetin and rutin, respectively. The results of that study indicated a high content TFC in propolis of Ukrainian origin. According to C.-C. Chan, the TFC of three Taiwan samples, one Brazil sample, and two Chinese samples, ranged from 2.82 to 7.73% with reference to quercetin (20). Therefore, our studies are in line with the studies of C.-C. Chan et al. Chan et al. also provide the results for three propolis tinctures: the TFC was in the range of 1.02% to 1.47% with refe-

Figure 1. Differential spectra of rutin trihydrate (50 mg/L), quercetin dihydrate (21 mg/L), and the propolis tincture T5.

Table 2. Calculations of the total flavonoid content in the developed tinctures of propolis from south Ukraine.

Identification of tincture	Absorption maximum/ mean value±SD, nm	Rutin		Quercetin	
		TFC±SD in tinctures, mg/L, TFC ± RSD	TFC in propolis, %	TFC±SD in tinctures, mg/L, TFC ± RSD	TFC in propolis, %
T1	405.4, 405.3, 405.9/ 405.5	10411 ± 361 10411 ± 3.47%	11.0%	-	-
T2	402.2, 402.7, 403.4/ 402.8 ± 0.6	17612 ± 387 17612 ± 2.20%	17.61%	-	-
	In 4 months				
T3	403.7, 403.6, 404.6/ 404.0 ± 0.6	13109.58 ± 603.51 13109.58 ± 4.61%	13.11%	5056.42 ± 232.78 13109.58 ± 4.61%	5.06%
	403.8, 402.1, 402.5/ 402.8 ± 0.9	16294 ± 1195 16294 ± 7.34%	16.29%	-	-
	In 4 months				
T4	403.8, 402.8, 402.1/ 402.9 ± 0.9	13947.78 ± 737.62 13947.78 ± 5.29%	13.95%	-	-
	405.8, 405.1, 405.3/ 405.4 ± 0.4	14554 ± 1019 14554 ± 7.00%	14.60%	5377.74 ± 284.5 5377.74 ± 5.29%	5.38%
	In 4 months				
T5	406.1, 404.3, 404.4/ 404.9 ± 1.0	13022.67 ± 1146.25 13022.67 ± 8.80%	13.00%	4784.31 ± 421.12 4784.31 ± 8.80%	4.78%
	403.5, 403.8, 403.6/ 403.6 ± 0.2	18499.20 ± 0.52% 18499.20 ± 95.52	19.50%	6796.51 ± 1.75 6796.51 ± 0.52%	7.18%
T6	404.2, 403.4, 403.1/ 403.6 ± 0.6	17703.19 ± 1209.9 18499.20 ± 6.83%	17.70%	6503.86 ± 6.83% 6503.86 ± 444.51	6.50%

Table 3. Statistical analysis for comparison of the total flavonoid content (TFC) in the propolis samples.

No.	Comparable Samples		Comparable mean values of TFC, mg/g		Standart deviation (SD) of mean values		$\bar{X}_1 - \bar{X}_2$	S_p^2	t	Conclusion 1	Conclusion 2
			$\bar{X}_1$	$\bar{X}_2$	SD_1	SD_2					
1	T2	T2	17.61	13.11	0.387	0.604	4.50	0.258	10.87	H_0 is rejected	The two means are statistically significantly different. There is an influence of the storage of the tincture on the TFC with reference to rutin
2	T3	T3	16.29	13.95	1.196	0.738	2.34	0.988	2.90	H_0 is rejected	The two means are statistically significantly different. There is an influence of the storage of the tincture on the TFC with reference to rutin
3	T4	T4	14.60	13.00	1.022	1.144	1.60	1.177	1.81	H_0 is accepted	The two means are equal. There is no effect of the storage time of the tincture on the TFC with reference to rutin
4	T5	T6	19.50	17.70	0.101	1.209	1.80	0.736	2.57	H_0 is accepted	The two means are equal. The propolis sample is homogenous. There is no effect on the TFC with reference to rutin
5	T2	T5	17.61	19.50	0.387	0.101	1.89	0.080	8.22	H_0 is rejected	The two means are statistically significantly different. There is an influence of the propolis collection year on the TFC with reference to rutin (the same location)
6	T2	T3	17.61	16.29	0.387	1.196	1.32	0.780	1.82	H_0 is accepted	The two means are equal. The propolis sample is homogenous. There is no effect on the TFC with reference to rutin

rence to quercetin. However, these authors did not state the ratio of propolis to a final tincture. In our studies, the TFC of the tinctures was in the range of 0.48 to 0.68% with reference to quercetin ($4784.31 \text{ mg/L} \pm 8.80\%$ to $6796.51 \text{ mg/L} \pm 0.52\%$ with reference to quercetin).

Quercetin or rutin were chosen as commercially available reference standards. Moreover, a lot of authors use quercetin for the TFC determination in propolis samples by the aluminum spectrophotometric method (15, 20, 34, 35). Bakchiche et al. employed rutin (36). Furthermore, quercetin and rutin were revealed in propolis (37).

Al-Ani et al. state that the TFC of the Irish propolis was $2.86 \pm 0.2 \text{ mg}$ quercetin equivalents per gram of the propolis extract while the Germany and Czech Republic samples contain approximately 2 mg per gram of propolis (15). Moreover, the technology of the ethanol propolis extract preparation was similar to our one for such parameters (70% ethanol, a ratio of propolis to the extract was 1 to 10, 24 h of maceration). Wang X. et al. determined from 21 to 53 mg quercetin equivalent per gram of the ethanolic extract of propolis (the Brazilian, Chinese, Australian, and Korean propolis samples) (35).

However, we could not compare these results with our ones as computing was given for propolis extract after special sample preparation (extraction, ethanol evaporation, and dilution for the TFC determination). Furthermore, these authors compare results for propolis of different origins not giving absolute values of the TFC. The computing of TFC is more complicated comparing different results obtained with different standards, for example, quercetin and galangin. Falcão et al. give results for flavones/flavonols and flavanones/dihydroflavonols in the range of 5-114 mg galangin equivalents per g of propolis extract and 35-118 mg pinocembrin equivalents per g of extract respectively, while the TFC range from 50 to 232 mg/g (5).

Zarate H. et al. state about a high content in the propolis from Mexico (various areas of Guanajuato). The TFC was in the range of 13 to 379 mg of quercetin equivalents per g of propolis (1.3-39.9%) (34). Bakchiche et al. state about a low TFC (379 mg rutin per 100 g sample, namely 0.379% of rutin equivalents) in the propolis from the south of Algeria (36).

Therefore, the TFC of propolis depends on an analytical procedure of the TFC determination, including a wavelength for measurements, reference standard, origin, way of computing, etc. It seems that it is impossible to compare the results expressed in different equivalents.

The TFC of tinctures and recalculations for the crude propolis samples with reference to quercetin and rutin are given in Table 2.

In our studies we used the absorption maximum of the reaction mixtures of the tinctures with aluminum chloride for the TFC determination, namely, we measured the absorbance of the reaction mixtures of the tinctures and reference standards in their absorption maxima. Speaking about the spectra of the reaction mixtures of the tinctures with aluminum chloride, it can be seen that these mixtures showed the absorption maximum in the range of 402.8 nm to 405.5 nm (Table 2, Fig. 1). A hypsochromic shift of the tinctures compared to rutin ($\lambda_{\text{max}} = 412 \text{ nm}$) and quercetin ($\lambda_{\text{max}} = 426\text{-}427 \text{ nm}$) could be explained by the presence of flavones in propolis, especially luteolin, apigenin, chrysin, and their glycosides, having an absorption maximum in the range of 385-395 nm (2, 15, 16, 20, 28).

Chan et al., Al-Ani et al., and Zarate et al. used a wavelength of 415 nm for measurements (15, 20, 34). Wang X. used a wavelength of 435 nm. Al-Ani et al., Zarate et al., and Wang X. did not explain why they chose such a wavelength (15, 34, 35). Moreover, these authors used quercetin for computing TFC (15, 34, 35). Additionally, Al-Ani et al. did not conduct calculations for the TFC determination in the propolis samples. They just compare the TFC in samples of Irish, Czech, and Germanic origin according to their elaborated analytical procedure. Falcão et al. used also a wavelength 415 nm for measurements and galangin as a reference standard (15).

Therefore, on the one hand, we employed two reference standards for easier comparison of our results with ones obtained by other authors. On the other hand, we used rutin as a reference standard because of the proximity of its absorption maximum to one of the tinctures after adding aluminum chloride.

Null hypothesis testing was employed for the comparison of two mean values of the TFC per 1 g of propolis obtained for the tinctures which are differed in year of the propolis collection, data of the TFC determination, and preparation of the tinctures from the same sample of propolis, etc. In all the cases the null hypothesis (H_0) was tested that the difference is equal to zero, namely, two means were regarded the same at H_0 ($\mu_1 = \mu_2$) (Table 3).

As can be seen from Table 3, the year of the propolis collection has a significant influence on the TFC (line 5) while the part of the propolis sample used for the preparation of tinctures does not influence the TFC. Therefore, it can be concluded that

the propolis samples are homogenous (lines 4 and 6).

Additionally, it was observed that there is a noticeable decrease in the TFC after 4 months that needs further studies in order to set up a storage period of propolis tinctures. The statistical analysis showed that in 2 cases of 3 there is a decrease in the tinctures TFC during storage (lines 1–3).

Propolis is a beekeeping product rich in flavonoids with known antimicrobial activity. Our results of the antimicrobial activity of the propolis tinctures are provided in Table 4.

As can be seen from table 4, the propolis tinctures evaluated in this study showed antibacterial activity against Gram-positive bacterial pathogens: *S. aureus*, including MR *S. aureus*, *S. pyogenes* and *C. diphtheriae*. We observe the logical dependence

of an increase in the inhibition zones on a volume of the tinctures and chlorhexidine put on the discs. Our study is in line with studies of Al-Ani et al. (15). Irish propolis demonstrated significant bactericidal activity against Gram-positive bacteria (*S. pyogenes* and *Bacillus subtilis*) (15).

E. coli and clinical isolate *C. tropicalis* were highly resistant to the propolis tinctures. The inhibition zones were absent that completely coincided with studies of Al-Ani et al. who stated that *P. aeruginosa* and *E. coli* were highly resistant to the propolis extracts (Irish, Czech, and German origin). The ethanolic extracts of propolis from Ireland and Czech showed a good fungicidal effect. The minimum fungicidal concentrations were in the range of 0.1 mg/mL to 2.5 mg/mL, while propolis of German origin showed mostly fungistatic activity.

Table 4. Antimicrobial activity of four tinctures of propolis.

	Inhibitions zones diameter, mm										
Volume, µL	2	3	4	5	1	Chl	Pen	Ak	Pef	Tet	Amp
Staphylococcus aureus ATCC 25923											
20	11	10	9	11	0	14	34	24	28	26	-
40	11	10	10	11	0	15.5					
60	10	9	9	10	0	18					
MR Staphylococcus aureus											
20	11	11	11.5	12.5	0	15	0	24	25	25	-
40	12	12.5	12	13.5	0	17					
60	13.5	13.5	11	14	0	20					
Corynebacterium diphtheriae NCTC 10356											
20	10	10	10	10.5	0	15	27	26	28	26	26
40	11	11	11	11	0	18					
60	12	13	12	13	0	20					
Streptococcus pyogenes (two isolates)											
20	12	12	10.5	12	0	16	35				35
40	13	12	12	13	0	20					
60	15	15	15	16	0	24					
Candida tropicalis											
20	Inhibition zones are absent				0	9	-	-	-	-	-
40					0	12.5					
60					8	0					
Escherichia coli ATCC 25922											
20	Inhibition zones are absent				0	12	-	24	28	-	27
40					0	12					
60					0	25.5					

Chl - 0.12% solution of chlorhexidine digluconate, Pen - penicillin, Ak - amikacin, Pef - perfloxacin, Tet - tetracyclin, Amp - ampicillin; 1 - 70 % ethanol; 2, 3, 4, 5 - tinctures T6, T2, a purchased tincture in a pharmacy and T1, respectively. SD ranged from 0.5 to 2.0 mm. The inhibition zones of *C. tropicalis* are 24 mm for nystatin, 28 mm for fluconazole (there was a growth of small colonies in the inhibition zone), 20 mm for itraconazole, and 26 mm for miconazole.

Figure 2. Inhibition zones for MR *S. aureus* (A) and *S. aureus* ATCC 25923 (B): 1 – ethanol; 2, 3, 4, 5 – tinctures T6, T2, a purchased tincture and T1 (60 μ L); 6 – 0.12% chlorhexidine digluconate (in the center of the Petri dishes).

Our study also partly corresponded to the studies performed by Kujumgiev et al. with propolis from Bulgaria, Albania, Mongolia, Egypt, Brazil, and Canary Islands. Kujumgiev et al. revealed that propolis samples were not active against the *E. coli* and were active against *C. albicans* (19). According to Marcucci, the ethanol extract of propolis inhibited *Candida* and all tested dermatophytes (14).

It is worth noting that *C. tropicalis* demonstrated a partial resistance even to fluconazole despite the inhibition zone of 28 mm as there was a growth of small colonies in the inhibition zone. Thus, we can suppose that the propolis of Ukrainian origin does not possess antifungal activity against the used clinical isolate of *C. tropicalis*.

The ethanolic extracts of propolis of Brazilian origin have a more pronounced activity against Gram-positive bacteria (*Staphylococcus* sp. and *Streptococcus mutans*) and *Candida albicans* ATCC 10231, and a less evident activity against Gram-negative and *Candida albicans* FT2010 (23).

Przybyłek I. and Karpiński T.M. state about the greater activity of propolis against Gram-positive bacteria compared to Gram-negative ones (38).

The control experiments with 70% ethanol showed that it does not have any activity. This is obvious as 70% ethanol was previously evaporated from discs. The drawback of this study is the absence of the influence of ethanol. It does not express a real administration of propolis tinctures in real clinical conditions. It could be supposed that the antimicrobial activity of the combination of propolis and 70% ethanol would be stronger. The second drawback is overlooking hydrophobic propolis components with antimicrobial activity in the method of

diffusion in agar. Therefore, we consider these studies as preliminary or antimicrobial screening of the propolis tinctures.

We would like to note that similar antimicrobial activities were observed for the tinctures of different samples of propolis and TFC in the tinctures. It is supposed that there are some still unknown, but common for all types of propolis, compounds which induce antimicrobial activity not depending on the propolis origin. One more drawback of this study is the non-testing of the purchased tincture for TFC. However, this tincture and T1 were tested for the total phenolic contents, which were similar, respectively, 14200 mg/L and 15849 mg/L (with reference to gallic acid) (32). Moreover, we determined the total phenolic content of one more purchase tincture of the same manufacture (lot 20216) which was 16586 mg/L.

CONCLUSION

The TFC measured by the spectrophotometric method can be used as a key index for the quality evaluation of propolis and its tinctures for pharmaceutical usage. The TFC was determined using the reaction of forming a complex of flavonoids with aluminum chloride. Propolis of Ukrainian origin is rich in flavonoids. The tinctures of propolis exhibit antimicrobial activity against *S. aureus*, *S. pyogenes* and *C. diphtheria*. Our studies also confirmed the absence of the antibacterial activity of propolis against *E. coli* and *C. tropicalis*. These studies are ground for the development of sprays with antibacterial activity against *S. aureus*, *S. pyogenes* and *C. diphtheria* for the complementary therapy of infection disorders of the oral cavity.

Further studies will be directed at the synergic activity of propolis tinctures and known antimicrobials drugs, and the development and studies of antimicrobial sprays on the base of propolis tinctures for the oral cavity. Currently, allergic screening of the sprays with the propolis tincture and herbal preparations are being performed.

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Conflict of interest

The authors declare no conflict of interest.

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