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Antioxidant Profile of Buckwheat Honey from the Republic of Moldova

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Abstract: *The honey is a natural substance, whose therapeutic qualities have been developed in parallel with human evolution. Its antioxidant and antiinflammatory properties are due to the polyphenolic compounds. The purpose of this study is to identify and quantify the phenolic compounds and the FTIR spectrum from buckwheat honey from the Balti region, Republic of Moldova. The radical scavenging activity of honey was 83% in the DPPH• reaction system. Phenolic compounds from honey were identified by HPLC-MS method, the significant concentrations being lignans, catechins, isorhamnetin, rutin, apigenin and luteolin. FTIR analysis revealed both the carbohydrate profile in buckwheat honey and the presence of active compounds.*

Keywords*: buckwheat honey, polyphenols, flavonoids, antioxidant activity, FTIR spectrum*

1.Introduction

Since ancient times, honey has been used not only as a food, but also as a main ingredient, in both alternative medicine for treating gastrointestinal, respiratory, dermatological and also in cosmetology. The honey is produced by *Apis mellifera* bees by mixing nectar from plants with their own substances, followed by maturation in honeycombs [1].

The honey, with a percentage of 95% carbohydrates, is an important product through the content of biologically active products. The most important factors in determining the composition of honey are represented by the botanical origin, geographical location, time and conditions of harvest [2].

Buckwheat is a plant with an old tradition of cultivation due to the nutritional content and especially of the antioxidant compounds present. At the same time, buckwheat is used to support honey production, being considered one of the best plants to obtain it.

Buckwheat (*Fagopyrum esculentum* Moench) of the family *Polygonaceae*, is a pseudocereal that arrived in Europe through Russia at the end of the Middle Ages. It was cultivated to supplement the harvest of basic cereals, especially in regions with poor sandy, clay or acidic soil, being a very resistant plant, which is why insecticides are not needed. Buckwheat shows a flowering period of 3 to 6 weeks and a complete maturation in 11-12 weeks [3]. Plants produce many white flowers and bloom in late summer when other plants no longer bloom [4]. Therefore, buckwheat honey is a very important source of nectar for bees, being a major product of the cultivation of this plant. It is estimated that 1 ha of land cultivated with buckwheat can produce between 125-300 kg honey [5].

Many studies have shown that dark color honey is richer in phenolic compounds, which would imply a higher antioxidant activity [6,7].

Buckwheat honey, with a dark color and a distinctive flavor, is a unique food product, containing bioactive compounds derived from bees and plants, rich in flavonoids, with beneficial effects on the body. Studies have shown that, due to the high content of phenolic compounds, buckwheat honey exhibits antibacterial activity against *Staphylococcus aureus* and *Micrococcus luteus* on varicose ulcer wounds [8].Brudzinsky and colab. showed in a 2012 study published in Frontiers in Microbiology [9] the bactericidal effect of buckwheat on *E coli* and *B. subtilis* cells. The latest studies [10, 11] have

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demonstrated the antibacterial potential of the buckwheat honey in nosocomial infections including *Clostridium difficile*, due to the high concentration of phenolic compounds and the synergistic contributions of saturated sugars (~ 80%)

The rutin, a flavonol with cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective properties [10,13] is present in buckwheat in larger quantities than in other plants. The ability of honey to scavenge the superoxide anion is used both in the food industry and in the pharmaceutical industry.

The purpose of this study is to identify and quantify the main polyphenolic compounds present in buckwheat honey from the Balti, a region of Republic of Moldova, in 2012. This study is the first to perform an analysis of flavonoid compounds present in the buckwheat honey from the Balti region, Moldova.

The infrared spectroscopic analysis (FTIR,) was used to determine the specific spectral fingerprint, thus being able to construct a model capable of predicting the physico-chemical parameters according to the obtained results. Also, by analyzing the FTIR spectrum, one can appreciate the antioxidant capacity of the respective honey [14, 15].

2. Materials and methods

2.1.Material

The study was carried out on buckwheat honey purchased from beekeepers from Balti region, the Republic of Moldova, in 2012.

2.2. Chemicals and reagents

All standards used were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Organic solvents methanol and acetonitrile, HPLC grade, were purchased from Merck Romania; formic acid (98%), Tris(hydroxymethyl)aminomethane, acetic acid, ammonium acetate, ammonia, ultrapure water were purchased from Merck (Romania). Solid phase extraction (SPE) StrataX 200 mg 6m/L cartridges were purchased from Phenomenex (Romania). Absolute ethanol, Folin-Ciocalteu phenol reagent, $AlCl_3$, were purchased from Merck Romania. All other chemicals and reagents were of analytical grade.

2.3. Analysis of total polyphenols content

The total polyphenol content (TPC) was determined by spectrophotometric analysis, using T80 UV/VIS spectrophotometer and gallic acid as standard, according to the method described by the International Organization for Standardization (ISO, 14502-1) [16] . Honey (5 g) was diluted to 50 mL with distilled water. One millilitre of this honey solution was mixed with 5 mL of Folin-Ciocalteu reagent (1:10) and 4.0 mL of sodium carbonate solution (7.5% w / v). After keeping the tubes at room temperature for 60 min, the absorbance at the wavelength λ = 765 nm was read using a UV-Vis spectrophotometer (T80 UV/ VIS spectrophotometer) relative to a control sample (in which 1mL of the extract was replaced). with 1mL of distilled water). The total polyphenol content was expressed as gallic acid equivalents (GAE) in mg/100 g of plant material. The TPC was determined by interpolation of the sample absorbance against a calibration curve built with gallic acid standards (10, 20, 30, 40, 50 μg/mL in 10% ethanol) and expressed as milligrams of gallic acid equivalents (GAE) per 100 mg of honey (mg GAE/100 mg), using the calibration curve, $y=0.071x+0.006$, $R^2=0.0092$

2.4. Analysis of total flavonoid content

The total flavonoid content (TFC) was determined by spectrophotometric analysis, using T80 UV/VIS spectrophotometer and quercetin as standard, according to the method [17] based on the formation of a yellow flavonoid-aluminum complex and its absorbance reading at 430 nm. To 1mL aqueous extract was added 1 mL 2% $AlCl_3$ atentie (2.5% w / v), 2 mL CH_3COOH (10% w / v) and ethanol (70% w / v) to a volume of 10 mL. After 30 min the absorbance at the wavelength λ = 430 nm

is being read. Flavonoid content was expressed as quercetin equivalents, and calculated using the standard curve plotted on the concentration range 10 and 50 μg / mL of quercetin, using the calibration curve, y=0.0045x+0.0062, R^2=0.00978. All the analysis were performed in duplicate.

2.5. Determination of antioxidant activity by DPPH method

The determination of antioxidant activity by the DPPH method was performed according to the protocol of Brand - Williams with some modifications [18]. The stock solution was obtained by dissolving 0.024 g DPPH in 100 mL methanol, was kept in a refrigerator until further use. Prior to analysis, a dilution of 1:10 with methanol was prepared from the stock solution. For analysis, 3 mL of DPPH working solution was added to 0.1 mL extract (1 mg/mL) or the standard solution. The absorbance was measured at 517 nm for a period of 30 min. The absorbance was read at the T80 UV / VIS spectrophotometer, the color of the solution being proportional to the antioxidant concentration.

The percentage of DPPH inhibition was measured according to the following equation:

$$RSA\ (DPPH\ \%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control sample. The control contained 100 μL methanol in place of the sample and A_{sample} is the absorbance of the analyzed extracts.

2.6. Sample preparation

To a gram of honey, there were added 5 mL of extraction solvent, 1% HCl in methanol. The samples were vortexed for 5 min, sonicated for 30 min, centrifuged for 15 min at 3000 rpm and then filtered. The obtained extracts were stored in the freezer and were used to determine total polyphenols and total flavonoids. The extract prepared for the determination of total polyphenols was filtered through a 0.45 μm filter and injected into the HPLC-DAD-MS Agilent 1200 system.

2.7. Analysis of phenolic compounds by HPLC

HPLC-MS analysis of the phenolic compounds by the HPLC-DAD-MS method was performed using the Agilent 1200 HPLC system with DAD detector coupled with Agilent 6110 single quadrupole MS detector. Positive ionization ESI mode was used.

The separation of the phenolic compounds was carried out on a chromatographic column as it follows: Eclipse XDB C18, size 4.6x 150 mm, 5 μm, Agilent, at temperature: 25^0 C.

The mobile phase consisted of: Solvent A: Water + 0.1% Acetic acid / Acetonitrile (99/1) v / v

Solvent B: Acetonitrile + 0.1% acetic acid. The gradient used corresponds to the first 2 min mobile phase A 95%, B 5% followed by a linear increase of solvent B to 40% until min 18, linear growth of phase B up to 90% the next 12 min, maintenance for 2 min and return to 95% A and 5% B in the next minute with holding for column balancing 5 min. Total analysis time - 30 min with a mobile phase flow rate of 0.5 mL / min

Monitored wavelengths: 280nm, 340 nm

For MS analysis, the positive ESI ionization mode was used. The ionization parameters were: Capillary voltage: 3000 V; Temperature: 350˚ C, Nitrogen flow rate: 8 l /min.

Data acquisition was done in full scan mode in the mass range m / z: 100-1000. The identification of phenolic compounds in samples was done by comparing the retention times, the UV and mass spectra with those of the injected standards.

2.8. FTIR analysis

FTIR analysis was performed for honey, using the Magna-IR Spectrometer 350 equipment (Nicolet Instrument Corporation, USA), by recording spectra from 4000 to 700 cm^{-1} with a spectral resolution of 1 cm^{-1}, using the technique of Total Reflection Attenuation (ATR - Total Reflectance Attenuator)

3. Results and discussions

TPC of buckwheat honey was 55 ± 0.6 mg GAE / 100 g sample. TPC is lower than Chinese honey (149.8 mg GAE / 100g [19], 169 mg GAE / 100g [20], or Polish honey (100 mgGAE / 100g [21] 87.28 mg GAE / 100g) [22].

TFC of buckwheat honey was 47 ± 0.8 mg QE / 100 g sample. TFC of buckwheat honey was about 10 times higher than Polish buckwheat honey (3.23 mg QE/100 g of honey)[23]. Compared to other varieties of honey, buckwheat has a much higher TFC.

The antioxidant activity of buckwheat honey was conducted by DPPH assay, which is one of the most stable free radical. The antioxidant activity of buckwheat honey (expressed as % of inhibition) was 83% . The obtained result can be compared to the study of [24], where the DPPH radical reaction system measured was 87.28% [22]. Compared to the current study, Dzugan and colab. [25]. described similar values (76.42-82.41%) for Polish buckwheat honey.

Table 1 shows TPC, TFC of honey in our study compared to other honey varieties.

Tabelul 1.TPC, TFC of honey in our study compared to other honey varieties

Honey	TPC mg GAE / 100 g honey	TFC mg QE / 100 g honey
Buckwheat (our study)	55 ± 0.6	47 ± 0.8
Dill [26]	32.5 ± 1.1	4.1 ± 0.2
Parsley [26]	28.3 ± 0.9	3.6 ± 0.2
Orange Bossom [26]	19.1 ± 0.1	1.7 ± 0.1
Rubus [27]	33.9 ± 15.4	1.4 ± 0.3
Wild rose [28]	11.6 ± 0.19	0.16 ± 0.03
Eucalyptus [28]	17.5 ± 1.16	4.13 ± 1.03
Orange bossom [28]	7.45 ± 0.66	1.73 ± 0.39
Lavender [29]	53.39 ± 23.34	2.20 ± 1.54
Astragalus [29]	43.63 ± 20.66	0.86 ± 0.49

Following the HPLC-MS analysis performed in the present study, the following flavonoid compounds were identified Table 2, Figure 1.

Table 2. Retention time, wavelength characteristic of maximum absorption and ion monitored in ms for phenolic compounds identified in samples

No. Peak	Retention time t_R (min)	$[M-H]^+$ (m/z)	UV λ_{max} (nm)	Compound
1	3,2	**379,** *363*	280	Hidroxisecolariciresinol *(lignan)*
2	4,7	**307,** *202*	280	Epigallocatechina (EGC)
3	5,5	**459,** *202*	280	Epigallocatechingallat (EGCG)
4	7,0	**459,** *202*	280	Gallocatechingallat (GCG)
5	9,4	**269,** *183*	240,300	Coumestrol *(isoflavonoid)*
6	12,1	**291,** *202*	280	Catechin
7	12,9	**363,** *247, 163*	280	Secolariciresinol *(lignan)*

8	14,0	**449,** *287*	270,350	Orientin
9	14,5	**449,** *287*	270,350	Isoorientin
10	15,4	**433,** 271	270,340	Vitexin
11	16,1	**611**, *475, 303*	250,360	Rutin
12	16,4	**493**, 475, *317*	250, 320	Isorhamnetin glucuronid
13	17,7	**435,** *303*	270, 330	Quercetin arabinosid
14	18,1	**449,** *433***,** *303*	270, 330	Quercetin ramnosid (Quercitrin)
15	21,9	**303**	260,370	Quercetin
16	23,4	**317**	260,370	Isorhamnetin
17	24,3	**287**	240,330	Luteolin
18	25,2	**271**	240, 320	Apigenin

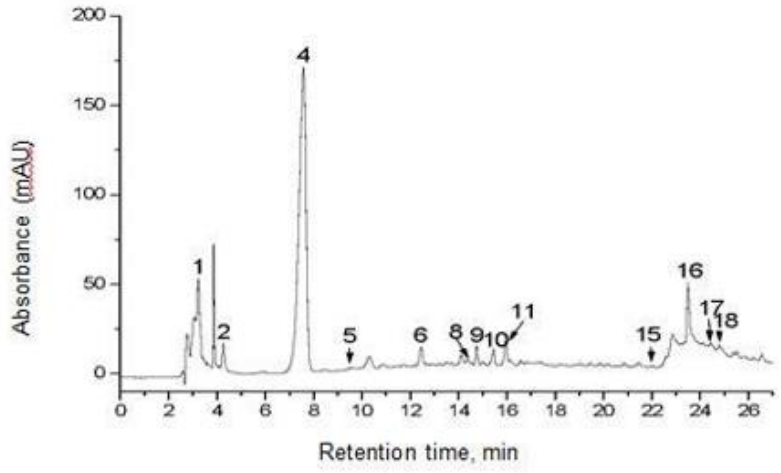

Figure 1. 280 nm chromatogram peak 1-hydroxisecoisolariciresinol; 2-EGC; 4-GCG; 5-coumestrol; 6-catechin; 8-orientin; 9-isoorientin; 10-vitexin; 11-rutin; 15-quercetin, 16-isorhamnetin; 17-luteolin; 18-apigenin

Flavonoid quantification, Table 2, was performed based on the calibration curves drawn for each compound, Table 4. The linearity of the method was demonstrated by the correlation coefficient that was for all the compounds analyzed between 0.99-1.

Table 3. Amount of flavonoids excreted in µg/g sample

Compound	**µg/g sample**
Hydroxisecoisolariciresinol	97.40
EGC	28.82
GCG	594.46
Coumestrol	4.60
Catechin	18.59
Orientin	10.40
Isoorientin	11.94
Vitexin	12.96
Quercetin	1.10
Rutin	19.44
Isorhamnetin	131.17

Luteolin	17.39
Apigenin	18.76

Table 4. Analytical performances of the method used for the quantification of individual flavonoid compound in honey

Validation parameters	Calibration equation	R	LOD µg/g	LOQ µg/g	Precision (RDS, %)		Repetability (RSD, %)
					intra-day	inter-day	
Hidroxisecolariciresinol	Y=11307X+4493	0.992	10.40	31.52	3.15	2.85	4.31
Epigallocatechina	Y=11198X+2849	0.993	9.65	29.24	2.49	2.62	3.56
Epigallocatechingallat	Y=3240X-3511	0.990	16.84	51.03	2.36	4.1	2.82
Gallocatechingallat	Y=8671X+2133	0.994	18.21	55.18	1.89	2.36	4.27
Coumestrol	Y=4735X-2771	0.995	0.82	2.48	2.23	3.04	3.95
Catechin	Y=1435X+305	0.993	0.35	1.06	2.81	5.01	3.97
Secolariciresinol	Y=1001X-261	0.998	1.65	5.00	2.65	3.25	4.04
Orientin	Y=5100X-4382	0.998	2.31	7.00	3.45	3.89	5.01
Isoorientin	Y=1294X+525	0.991	1.15	3.48	4.25	3.87	3.26
Vitexin	Y= 7555X+2105	0.998	2.47	7.48	3.29	4.32	3.57
Rutin	Y=3022X+156	0.996	11.02	33.39	2.47	2.65	2.12
Isorhamnetin glucuronid	Y=43279X-1900	0.996	3.45	10.45	2.43	3.16	5.04
Quercetin arabinosid	Y=74639X+2708	0.994	8.31	25.18	1.26	2.08	3.44
Quercetin ramnosid	Y=54104X-5319	0.991	9.03	27.36	3.11	3.15	2.27
Quercetin	Y=53615X-1292	0.992	0.32	0.97	4.48	1.58	32.23
Isorhamnetin	Y=69535X-2208	0.993	10.5	31.82	3.04	4.28	4.98
Luteolin	Y=14473X-4389	0.995	4.10	12.42	2.97	3.18	3.61
Apigenin	Y=24574X+5685	0.995	6.02	18.24	3.65	3.78	3.18

Note: LOD, limit of detection; LOQ, limit of quantification.

The floral origin of nectar and pollen determines the content in phenolic compounds and consequently the bioactive properties of bee honey [30].

It is observed that the highest concentrations were obtained for GCG, EGC, flavonoids that have not been found in other types of buckwheat honey [19, 28]. Small amounts of catechins have been identified in heather honey (23.07 µg / g), lavender honey (7.85 µg / g), chaste tree (1.87 µg / g) [29]. This fact shows the possibility of buckwheat honey to be used in different nutraceuticals, knowing the role of catechins in inhibiting bacterial growth [31], the effect of hypocholesterolemia [32, 33, 34] by lowering cholesterol absorption and increasing fecal excretion and decreasing ROS induced cytotoxicity through intracellular scavenging [35].

Also, a significant concentration of isorhamnetin was identified (131.17 µg/g sample) in buckwheat honey. The presence of isorhamnetin, but in much smaller amounts, has been reported by Can [29],in honey acacia (3.25 µg/g sample) and by Yao in eucalyptus honey (4-1.1 µg/g sample) [36]. Studies have shown that isorhamnetin exerts strong anti-inflammatory and immunosuppressive activities, and can be used to treat both chronic inflammation and autoimmune diseases [37, 38, 39].This honey contains a number of flavonoids. For example, quercetin, orientin, isoorientin, vitexin, luteolin, apigenin, isorhamnetin are also present in other varieties [19, 28, 29, 40].

Of these, the highest concentration is isorhamnetin, of 131.17 µg / g sample, which may confer particular properties, as this flavonoid has important antioxidant, antibacterial, antiinflammatory, hepatoprotective, cardioprotective [41] activities. Rutin was found at a concentration of 19.44 µg/g sample. In other types of buckwheat honey this flavonoid has not been identified [19, 21, 42].

It was also observed that other flavonoids such as chrysin, kaempferol, myricetin, galangin [43, 44, 45] were not detected in buckwheat honey from the present study.

Of the total phenolic compounds, 80% are flavonoids, the rest being mainly lignans, namely hydroxisecoisolariciresinol, in a surprisingly high concentration, 97,40 μg / g sample and coumestrol, 4,60 μg / g sample. The level of the main phenolic acids (caffeic, p-hydroxybenzoic, ferulic) present in other studies [20, 21, 42] are in insignificant quantities. Although in a small amount, coumestrol may exert its stimulation effects on the estrogen receptor, inhibiting adipocyte differentiation [45], known that after pass through the gastric passage, the structure of coumestrol does not change [46, 47], and may also act at small concentrations.

ATR-FTIR infrared spectroscopy is a rapid method used for the qualitative characterization of honey. The analysis of the spectrometric fingerprint of honey by determining the chemical bonds specific to the characteristic chemical compounds [48].

In Figure 2 and Table 5 are presented to the FTIR absorption bands, together with the corresponding vibration allocation for buckwheat honey.

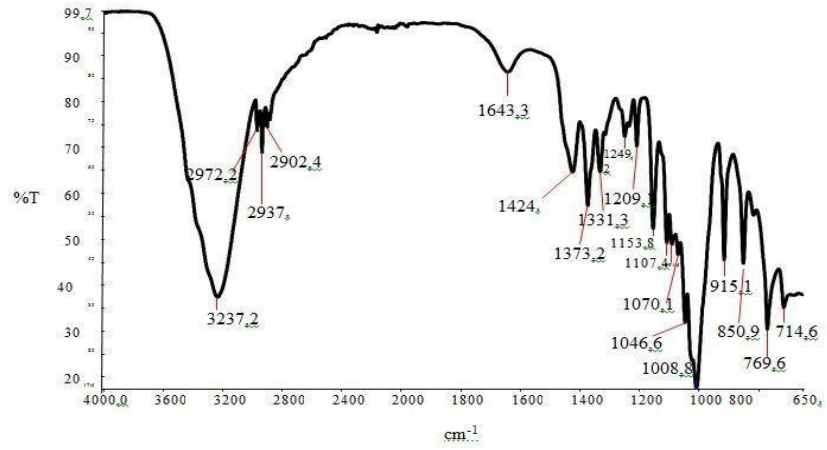

Figure. 2. The absorption bands of the FT-IR spectrum of buckwheat honey

The spectral domains most often used in honey analysis are those within the fingerprint region, 700-1500 cm^{-1}, which corresponds to the absorption of monosaccharides, such as glucose and fructose and disaccharides, sucrose [49, 50] as well as the region 2500-4000 cm^{-1}, which characterizes the antioxidant capacity [51].

Table 5.Allocation of the corresponding vibrations for the honey of spectrally made 700-4000 cm^{-1}

Position of the band (cm^{-1})	Type and origin of vibration
769,6	δ (C-H) from the structure of carbohydrates
850,9	
915,1	
1008,8	ν (C-O) in the (C-OH)group or ν (C-C) group in the structure of carbohydrates
1046	
1070,1	ν (C-O) in the (C-O-C) group
1107,4	
1153,8	ν (C – H) in carbohydrates or / and ν (C – O) in carbohydrates
1209	
1249	
1331,3	δ (-OH) in the (C-OH) group
1373,2	
1424	δ (O-H) in the (C-OH) group and δ (C-H) in alkene

1643,3	ν (C=O) or ν (C=C) from polyphenols [52, 53]
2902,4	ν (C-H), ν(O-H) or/and ν (NH3) from free amino acids to carboxylic acids or polyphenols [54, 55],
2937	
2972,2	
3237,2	ν (O-H) polyphenols

ν - stretch vibrations, δ - deformation vibrations,

The intense signal was observed at 3237.2 cm^{-1} where a very large absorption band, characteristic of the O-H bonds, due to the presence of phenolic compounds, was highlighted. Tewari et al. (2005) and Matysek (2018) studied the FTIR fingerprints of several types of honey (buckwheat, clover, alfalfa, carrot, wildflower, orange blossom, sunflower) and found that the absorption band in the area of 3200 cm^{-1} is the largest for buckwheat, which reveals the highest amount of polyphenols. The FTIR spectral profile of buckwheat in this study is similar to that of buckwheat in Kędzierska-Matysek's study [48].

4. Conclusions

Phenolic compounds and antioxidant activity can be considered some important factors for honey quality and its functional properties.

Given the results obtained from the analysis of polyphenolic compounds for buckwheat honey used in this research, it is possible to notice that the differences in the content of polyphenols and flavonoids, depending on the geographical area and the floral source.

The results of the study contribute to the knowledge of the major composition of phenolic compounds from buckwheat honey from the Balti region, Republic of Moldova and allow comparison with other honey samples from other countries. Thus, the buckwheat honey from our study is highlighted by much higher amounts of flavonoids compared to phenolic acids. There are high concentrations of GCG, EGC, isorhamnetin.

These determinations made on buckwheat honey from the Republic of Moldova offer opportunities for its use both as a main ingredient in many pharmaceutical and cosmetic preparations, as well as a valuable food for sustaining health.

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