BLACK SEED OIL AS AN ADDITIVE TO HONEY

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ABSTRACT: *Nigella sativa is a well-known plant with rich history and religious heritage. Black seed is mostly composed of aminoacides, fat acids, proteins, sterols, different essential metals, carbohydrogens, fats and water. Black seed oil is coloured yellow, which implicates presence of yellow pigments (carotenoids). Oxidative stability of black seed oil can last up to 55 hours, considering that oil with more polyphenols is more stable. In this study was determined antioxidative capacity of black seed oil, honey and black seed oil and honey mixture using FRAP and DPPH methods. Methanol was used as solvent. Results show that adding black seed oil to honey increases antioxidative capacity of honey***.**

KEYWORDS: honey, black seed oil, antioxidative capacity

INTRODUCTION

Use of herbs in prevention and treatment of different medical conditions is almost as old as human race. Herbal product are made by processing plants using extraction, destillation, percolation, refinement, concentration or fermentation.Plant are enrolement in food chain, chemical elements cycles and enviroments quality. They have bees used as drugs for different conditions treatement which is based mostly on their chemical composition. Pharmacologicly active ingredients are usually part of secondary metabolites, because they are rusult of metabolic changes of primary metabolism products during ensyme catalysis.

LITERATURE/THEORETICAL UNDERPINNING

Nigella Sativa L.

Nigella sativa L. is an edible, annual flowering plant in the family Ranunculaceae, native to south and southwest Asia, middle Europe, middle and west region of Saudi Arabia. N. sativa grows to 20–30 cm tall, with finely divided, linear leaves. The fruit is a large capsules consisting of three to seven united follicles, and in the follicles are located small black seeds of a strong aromatic scent and flavor.bThe most common phenolic compounds present in Nigella sativa L., which are attributed to antioxidant properties, are thymol, carvacrol, thymoquinone, thymohidroquinone, dithymoquinone, gallic acid, and then 3.4-dihydroxybenzoic acid, vanilic acid, chlorogenic acid, syringic acid, caffeic acid, benzoic acid, cinnamon acid, catechine, epicatechin. In addition to these compounds, the seed of the plant is rich with other various ingredients, among which stand out: amino acids, fatty acids, proteins, sterols, various metals, carbohydrates, fats and water.

The seeds of black cumin is due to their chemical composition found wide application in medicine. The seeds are digestive stimulants, diuretics, antipyretics, analgetics, and have antimicrobial, antitumorous, antioxidant and other properties.
Most of the therapeutic effect is attributed to the presence of thymoquinone, which is the main bioactive component of the essential oil.

Honey

Honey is a part of traditional medicine thanks to its dietary and pharmaceutical properties since ancient times. Honey is a thick, viscous, liquid or crystallized product of the honey plants nectar or secretion of living parts of plants.The composition of honey and its properties primarily depend on botanical origin, but there is also influence of geographic position, climatic conditions, types of bees, processing conditions and further handling of honey.Honey has a very variable composition, so specifying the average composition of honey is limited. Some of the honeys components depends of honey bees, some of the honey plants, while some are formed or changed during the maturation of honey.

Components that make up the majority of honey are carbohydrates and water, while, in smaller quantities, are present organic acids, mineral substances, pigments, vitamins, proteins (including enzymes), the components of the aroma and phenolic components thanks to which honey has and antioxidant property. In most cases, in honey is a larger amount of fructose than glucose, but there are some types of honey in which glucose prevails. The relationship of fructose and glucose is one of the parameters identification of honey and its value is usually greater than 1.

Research has shown that honey has a bactericidal and bakteriostatic effect on the many bacteria, many of which are pathogens. Last researches suggest that the consumption of honey alone or with other antioxidant beverages significantly increases the capacity of antioxidants in human serum.

Antioxidative Capacity

There is a growing interest in antioxidants, especially for those intended to deal with the flow of the harmful effects of free radicals in the human body, as well as to prevent the deterioration of fats and other ingredients of food products. In both cases, preferred are antioxidants from natural, and not from synthetic sources.

Free radicals and other reactive oxygen compounds induce the oxidation of proteins, amino acids, unsaturated lipids and DNA. The human body has a defense mechanism against the free radicals present in almost all cells. It is possible for imbalance to happen between free radicals and their elimination from the body by the antioxidative system. This imbalance leads to a phenomenon known as oxidative stress. The balance between free radicals and antioxidants can be restored from external supplies of anti-oxidants.

Numerous mechanisms for the removal of free radicals and/or preventing their production can be grouped in different ways. One of them is the assignment in the first, second and third level of defensive mechanisms to overcome the reduced oxygen intermediates, (ROS) – their removal and the consequent reduction in the harmful effects. The first (primary) level of protection are the enzyme and nonenzyme systems

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that completely prevent the formation of free radicals within cells. The other (secondary) level of antioxidant protection is formed by systems that operate in normal and increased formation of radicals - conditions. In this group are protein-specific oxidoreductases, protein-ADP-ribose transferase, ATP and Ca-independent protease inhibitors. The enzyme systems involved in the repair of oxidative damaged macromolecules make the third (tertiary) level of antioxidant protection. In addition to the primary, secondary and tertiary, antioxidants are, for easier consideration, classified as enzyme, nonenzyme and extracellular.

Essential oil of Nigella sativa L. has antioxidant activity that can come from thymoquinone, carvacrol, trans-anethole and 4-terpinolene, because these compounds have the ability of "capturing" free radicals. Thymoquinone is notable due to its bioactive and antioxidant qualities. In N. sativa oil, thymoquinone is also present in its other forms such as dithymoquinone and dihydrothymoquinone (Figure 1).

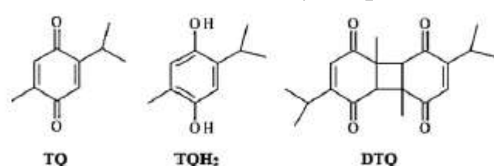

Figure 1. Structural formulas of thymoquinone, thymohidroquinone and dithymoquinone.

Strong ability to "capture" anion radicals superoxide and reactive oxygen species occurs according to the possible mechanism given in Figure 2.

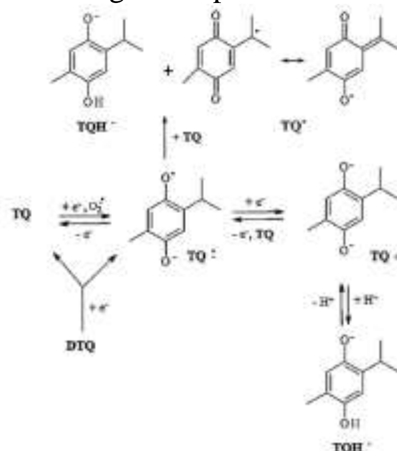

Figure 2. Possible mechanism of antioxidative affect.

Experimental data show that thymoquinone has a dual nature, and that it can act as electron – acceptor and as the H-atoms donor. As an electron donor, thymoquinone acts as the other biological quinones, such as CoQ0 and CoQ10, so the main activity of the superoxides „capturing“ is done via an electron-transfer mechanism.

Antioxidant properties of honey and preventive effects against different diseases, such as tumors, are mostly credited to phenolic components, precisely, flavonoids, phenolic acids and phenolic acid derivatives, depending on their concentrations or ratios.

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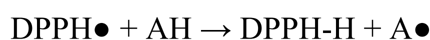
METHODOLOGY

In this study, the antioxidant activity of natural honey, black cumin oil, black cumin oil and honey mixture (in which the honey was dominant component) were investigated and determined. Also, in this paper was determined the content of active components: gallic acid, thymoquinone and total phenols in all samples. The goal was to show that the black seed could be used as an additive to honey that will improve the antioxidant properties of honey.

The relative antiradical activity of all samples is determined by using a stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) which is often used in the assessment of the antioxidative activities. Also, FRAP method was used, which is based on the antioxidants ability that by donating electrons in acidic medium (pH 3.6) reduces the yellow ferry complex iron (Fe^{3+}) with TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) into blue colored complex of Fe^{2+}-TPTZ. Intensity of resulting blue color was measured spectrophotometrically at 593 nm. The intensity of the color is proportional to the reductive capacity of antioxidants. The total amount of active components (gallic acid and thymoquinone) were determined by HPLC methods by Kardani et al., 2013., and Isik et al., 2017., while the total phenols were determined spectrophotometrically using the Folin – Ciocalteu reagent with gallic acid as standard.

DPPH radical (2,2-diphenyl-1-picrylhydrazyl) is one of the rare stable forms of nitrogen radicals. In the reaction with antioxidants, DPPH radical is reduced in hydrazine. The reduction capability of DPPH radicals "capturing" is monitored by measuring the changes in absorbance at 515-528 nm. DPPH radical absorbs light at 515 nm. In the reaction with antioxidants or free radicals, absorbance decreases and its intensity is measured spectrophotometrically.


DPPH● + AH → DPPH-H + A●

DPPH● + R● → DPPH-R

One of the ways of expressing the antioxidative capacity of a sample is through IC50, which indicates the concentration of antioxidants that is required for the reduction of the initial concentration of DPPH ● by 50%.
DPPH method (described in the work of Nameer Khairullah Mohammad et al., 2016.) was used to determine the antioxidative capacity of honey, black seed oil, and oil and honey mixture.

For the determination of antioxidant capacity of the above-described samples, modified FRAP method (described in the work of Cilović et al., 2015.) was used.
To 0.1 mL of sample 3 mL of freshly prepared FRAP reagent was added. The mixture was incubated for 30 minutes and absorbance was measured on 593 nm. As a blind test has been used solution that was made by mixing 3 mL FRAP reagent and 0.1 mL of methanol. For constructing a calibration curve 11 solutions of $FeSO_4$ x $7H_2O$ were prepared in concentration range from 0.05 to 1.0 mM.

The concentration of antioxidants in the samples was calculated based on calibration curves equation and the results are expressed in µmole Fe^{2+}/L.

For both methods, same samples of honey, black seed oil, and honey and black seed oil mixture are prepared:
- a solution of honey in methanol: 5.55 g of honey/25 mL methanol
- solution of oil in methanol: 1.96 g of oil/25 mL methanol
- solution of a mixture of honey and oil in methanol: 3.07 g honey and 2.01 g/oil 25 mL methanol

All solutions were sonicated for 3 hours, and for analysis HPLC further filtered through 0.45 μm filter.
Solutions of above described samples were made:
Solution 1: 6 mL stock solution / 10 mL methanol
Solution 2: 5 mL solution / 10 mL methanol
Solution 3: 5 mL solution 2 / 10 mL methanol
Solution 4: 5 mL solution 3 / 10 mL methanol

RESULTS/FINDINGS

RESULTS OF ANTIOXIDATIVE CAPACITY (DPPH METHOD)

To 0.25 mL of the sample solution was added 1.75 mL of freshly prepared DPPH reagent. Mixture was incubated for 30 minutes in dark, room temperature, and measurement was made at 515 nm. As a control solution, mixture of 1.75 mL DPPH reagent and 0.25 mL of methanol was prepared. Based on the measured absorbance, the degree of inhibition (%) was calculated.

$\% = \frac{A_C - A_S}{A_C}$ Where is:

A_C – absorbance of control,
A_s – absorbance of sample.

Based on calibration curves equation, IC_{50} value was calculated. The quantity of the sample, which reduces absorbance of DPPH solution for 50%, is taken as the final point of measuring antioxidant activity. Therefore, antioxidative capacity results are expressed in mg/mL.Graph of inhibition in function of sample concentration was used for calculating IC_{50} values. The results obtained by the DPPH method are expressed as IC_{50} (mg/ml), as the concentration of the sample (mg/ml) required for 50% reduction on the initial values of $DPPH^{\bullet}$. Lower value of IC_{50}, greater the antioxidant capacity of the analyzed sample. The values of obtained results are given in Table 1.

Table 1. Results of DPPH analysis.

Solution	IC_{50} (mg/mL)
Honey in methanol	63.09
Black seed oil in methanol	2.10
Honey and black seed oil mixture in methanol	5.86

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RESULTS OF ANTIOXIDATIVE CAPACITY (FRAP METHOD)

For constructing a calibration curve 11 solutions of $FeSO_4$ solution x $7H_2O$ were prepared in concentration range from 0.05 to 1.0 mM and obtained equation was y = 2.552x – 0.023 (R^2 = 0.9938).
The concentration of antioxidants in samples was calculated based on the equation of the calibration curve and final volume of the sample. The results are expressed in µmole Fe^{2+}/L and are presented in Table 2.

Table 2. Results of FRAP analysis.

Solution	Concentration (mg/mL)	FRAP (µmol/L)
Honey in methanol	7.16	466.38
Black seed oil in methanol	2.53	191.66
Honey and black seed oil mixture in methanol	6.55	1678.27

RESULTS OF CHEMICAL ANALYSIS OF CONTENT OF GALLIC ACID AND THYMOQUINONE IN SAMPLES OBTAINED BY HPLC METHOD

Quantification of gallic acid in samples was made by method of high pressure liquid chromatography. Chemicals and reagents used: acetonitrile (Honeywell, HPLC grade), methanol (Honeywell, HPLC grade), o-phosphoric acid (Sigma-Aldrich, p.a.), purified water. Analyse was performed using HyperClone BDS C18 250 x 4.6 mm, 5 µm column and mobile fase purified water / acetonitrile (80/20 V/V %), pH = 5. Flow rate was 1.0 mL/min and volume of injection was 20 µL.
To determine the content of gallic acid approximately 7.06 mg of gallic acid WS was dissolved in 25 mL methanol (0.2824 mg/mL). From this stock solution were made solutions in concentration range 0.00353 mg/mL – 0.1412 mg/mL.
In Figure 3 is shown the chromatogram one of the standard solutions (0.01765 mg/mL).

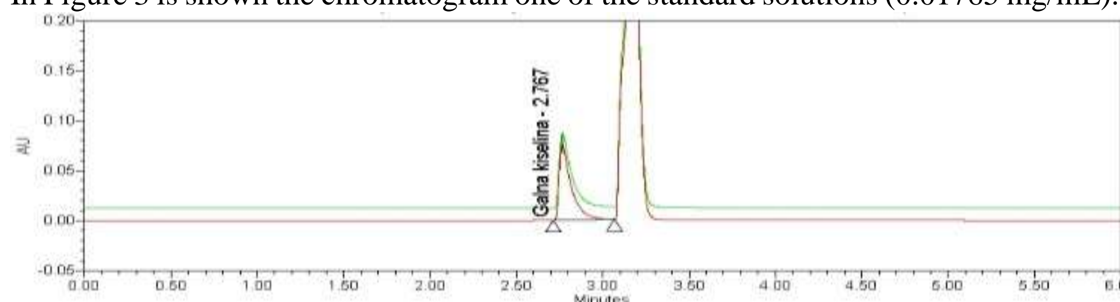

Figure 3.Chromatogram of gallic acid standard solution.

Chromatograms of diluent and mobile phase were recorded and it has been proven that there is no interference on the retention time of gallic acid peak.
Based on the area of gallic acid peaks in standard solutions, calibration curve was constructed and the equation was $y = 4x10^7x– 746407$ (R^2 = 0.9789).
The concentration of gallic acid in samples was calculated based on the equation of the calibration curve and final volume of the sample. The results are expressed in mg/g of sample and are presented in Table 3.

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Table 3. Gallic acid content.

Solution	Gallic acid (mg/g)
Honey in methanol	0.14
Black seed oil in methanol	0.24
Honey and black seed oil mixture in methanol	0.13

To determine the content of thymoquinone approximately 7.88 mg thymoquinone WS was dissolved in 25 mL of 96% ethanol (0.3152 mg/mL). From the stock solution thymoquinone WS in 96% ethanol, solutions were made in concentration range 0.0003 mg/mL – 0.1576 mg/mL.
Chemicals and reagents used: 2-propanol (Honeywell, HPLC grade), methanol (Honeywell, HPLC grade), purified water.
Analyse was performed using Zorbax Eclipse XDB-18; 4.6 x 150 mm, 5 µm column and mobile fase purified water / methanol / 2-propanol (50/45/5 V/V/V %), pH = 3.017. Flow rate was 0.9 mL/min and volume of injection was 10 µL.
In Figure 4 is shown the chromatogram one of the standard solutions (0.0788 mg/mL).

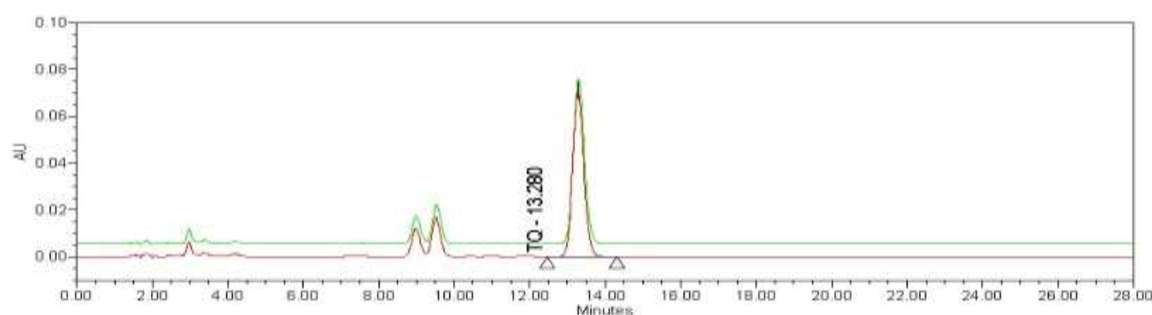
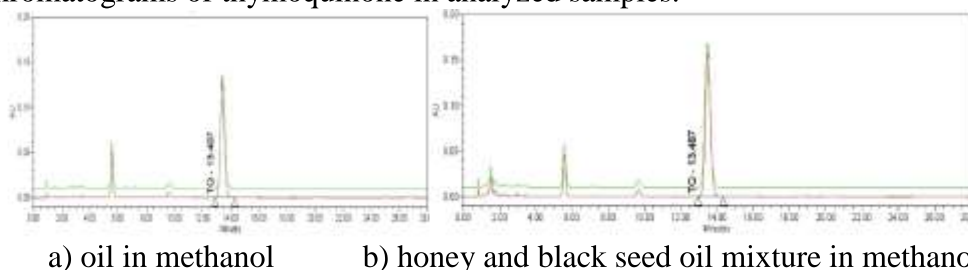


Figure 4.Chromatogram of thymoquinone standard solution.

Chromatograms of diluent and mobile phase were recorded and it has been proven that there is no interference on the retention time of thymoquinone peak. In Figure 5 are shown chromatograms of thymoquinone in analyzed samples.



a) oil in methanol b) honey and black seed oil mixture in methanol

Figure 5. Chromatogram of thymoquinone.

Based on the area of thymoquinone peaks in standard solutions, calibration curve was constructed and the equation was $y = 2x10^7x + 14002$ ($R^2 = 0.9998$).

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The concentration of thymoquinone in samples was calculated based on the equation of the calibration curve and final volume of the sample. The results are expressed in mg/g of sample and are presented in Table 4.

Table 4.Thymoquinone content.

Solution	Thymoquinone (mg/g)
Black seed oil in methanol	1.78
Honey and black seed oil mixture in methanol	0.86

TOTAL PHENOLS CONTENT

Total phenols were determined spectrophotometrically using the Folin – Ciocalteu reagent with gallic acid as standard. Chemicals and reagents used: Folin – Ciocalteu reagent (diluted with water 1:1), gallic acid WS, methanol (Honeywell, HPLC grade), Na_2CO_3 (Sigma-Aldrich, p.a.), purified water, 7% solution of Na_2CO_3.
0.5 mL sample solution, 0.5 mL of the Folin – Ciocalteu reagent (diluted with water 1:1, v/v) were placed in the centrifuge tube of the smaller volume. Solutions were left to stand for 5 minutes at room temperature. In each solution is then added 10 mL of 7% Na2CO3 (w/v). All of the prepared solution were mixed, centrifuged for 5 minutes at 3000 rpm and left to stand for 1 h in the dark at room temperature. Absorbances were measured spectrophotometrically at wavelength of 765 nm (Thermo Fisher, Evolution, 100 UV/VIS Spectrophotometer) in relation to a blind test. Blind test is prepared in the same way as the sample, but instead of 0.5 mL of the sample solution used is 0.5 mL of diluent.
Total phenos are quantified based on calibrations curves equation $Y = 5.1613x + 0.0095$ ($R^2 = 0.9999$)
Standard solutions were prepared in the same way as sample solutions using 0.5 mL of gallic acid solution.

During the wavelenght check, spectra of all of the solutions were recorded in the interval of 600 nm – 850 nm. Solution showed maxima absorbance at wavelenght 765 ± 2 nm and later measurements were made on that wavelenght.
Spectra of the blind test was recorded and it was proven that there were no interference of blind test on that wavelenght.
The obtained values are expressed as mg equivalents of gallic acid per gram of material (mg/g) and are presented in Table 5.

Table 5.Total phenols content.

Solution	Total phenols (mg/g)
Honey in methanol	0.004
Black seed oil in methanol	0.110
Honey and black seed oil mixture in methanol	0.028

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DISCUSSION

The results obtained by the DPPH method are expressed as IC_{50} (mg/ml), as the concentration of the sample (mg/ml) required for 50% reduction on the initial values of $DPPH^{\bullet}$. Relationship between the values of IC_{50} is given in Figure 6.

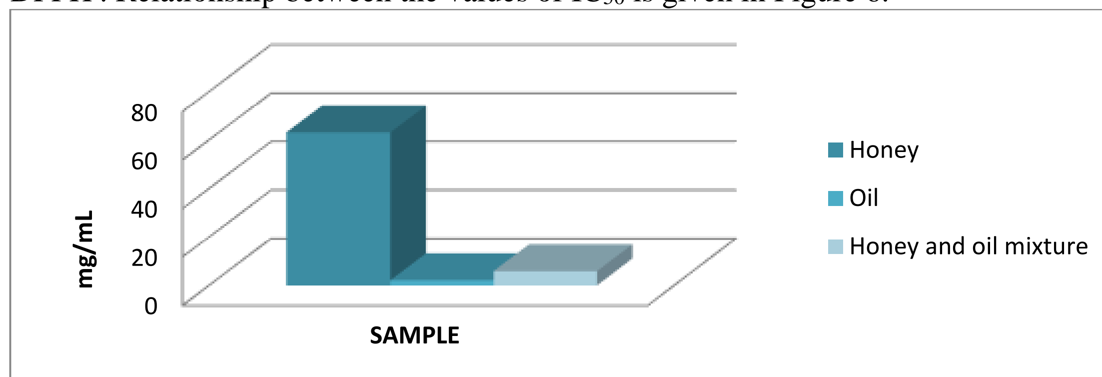

Figure 6.Antioxidative capacity of analyzed samples determined by DPPH method.

The results obtained by applying the FRAP method to samples of honey, oil and mixture are expressed in µmol Fe^{2+}/L and presented in Figure 7.

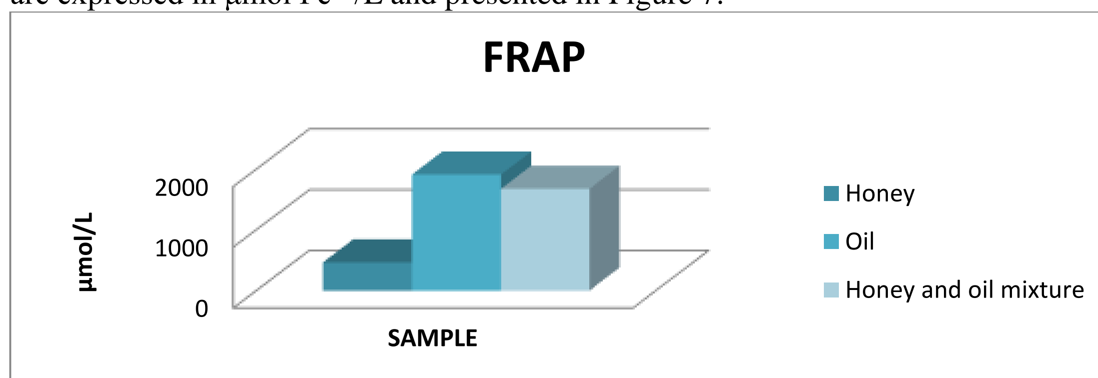

Figure 7.Antioxidative capacity of analyzed samples determined by FRAP method.

In Figure 8 the results are provided for the content of gallic acid, thymoquinone and the total phenolic compounds in the analyzed samples.

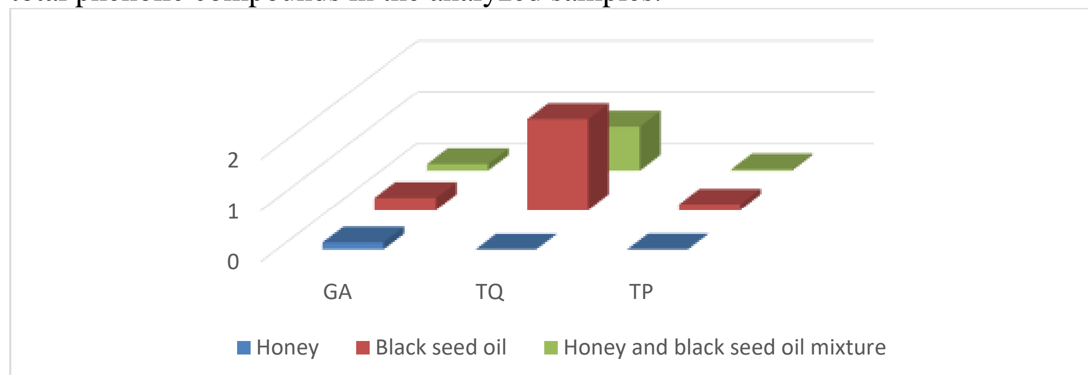

Figure 8.Content of gallic acid, thymoquinone and the total phenolic compounds in the analyzed samples.

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IMPLICATION TO RESEARCH AND PRACTISE

From the results obtained in DPPH method it is visible that the best antioxidant capacity has a solution of black seed oil, but it is also evident that the mixture of black seed oil and honey has a much higher antioxidant capacity than the honey.
Given that the smaller the quantity of the substance reduces large amount of Fe^{3+} indicates a better antioxidant capacity of the substance, from the results obtained in FRAP method, is seen that the addition of black seed oil to the honey (2:3) significantly increases the amount of reduced iron, or that the antioxidant properties of honey were significantly improved.

CONCLUSION

Black seed oil has shown a significant capacity for neutralization of free DPPH radicals, much more than pure natural honey, even more then oil and honey mixture. Therefore, it can be used as an antioxidant component in the form of the addition to the honey, and to prevent or decelerate oxidative stress caused by free radicals or as a potential natural antioxidant in the food industry instead of synthetic antioxidants. The results of the analysis on the content of active components showed that the mixture of honey and black seed oil in relation 3:2 has a higher concentration of total phenols in relation to pure honey and that the significant quantity of Thymoquinone has gone into the mixture.

FUTURE RESEARCH

Black seed oil obtained by cold extraction or by Soxhlet extraction can be used in the food industry due to potential its biological and pharmacological activity, which is necessary to examine in more detail. The results showed that black seed oil could be an important source for the production and application of food industry of nutritive supplements, or natural antioxidants. The results showed that the addition of the oil to the honey can improve antioxidant capacity of honey and that the food of the mild scent and pleasant taste is obtained.

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