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The Journal of Apitheraphy and Nature accepts English-language original articles, reviews, and letters to the editor concerning various fields of research. Main topics include:

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Investigating the Antioxidant Properties of Some Herbal Infusions During In Vitro Digestion

Bazı Bitkisel İnfüzyonların In Vitro Sindirim Sırasında Antioksidan Özelliklerinin İncelenmesi

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Abstract

In the present study, the content and antioxidant potential of phenolic compounds from five herbal infusions including *Aspalathus linearis, Sambucus nigra, Rhamnus frangula, Plantago lanceolata and Equisetum arvense* were analyzed. Additionally, simulated in vitro gastrointestinal digestion were conducted to predict the bioaccessibility of infusion phenolics. Accordingly, Rooibos infusion had the highest total phenolic (2191.18±93.39 mg GAE/100 g dw) and flavonoid content (1226.14±93.07 mg CE/100 g dw) as well as antioxidant capacity measured by CUPRAC (3124.12 mg TE/100 g dw) and FRAP (2634.95 mg TE/100 g dw) assays. On the other hand, the highest ABTS (2351 mg TE/100 g dw) and DPPH (475.19 mg TE/100 g dw) antioxidant capacity values were recorded for elderflower infusion. Based on the gastrointestinal digestion, retention of phenolics in the infusions was found to be variable througout mouth to intestine. It can be deduced from the results that those herbs can be considered as ~~a~~ valuable beverage alternatives with their high nutritional value.

Keywords: Herbal infusions, Antioxidant capacity, Bioaccessibility, Phenolic compounds

Özet

Bu çalışmada, *Aspalathus linearis, Sambucus nigra, Rhamnus frangula, Plantago lanceolata* ve *Equisetum arvense* olmak üzere beş bitkisel infüzyonun fenolik içeriği ile antioksidan potansiyeli analiz edilmiştir. Ayrıca, infüzyonlarda bulunan fenolik bileşiklerin biyoerişilebilirliklerini belirlemek amacıyla simüle edilmiş in vitro gastrointestinal sindirim gerçekleştirilmiştir. Sonuçlara göre, Rooibos infüzyonunun en yüksek toplam fenolik (2191.18±93.39 mg GAE/100 g ka), toplam flavonoid (1226.14±93.07 mg CE/100 g ka) ve antioksidan kapasiteye (CUPRAC, 3124.12 mg TE/100 g ka; FRAP, 2634.95 mg TE/100 g ka) sahip olduğu bulunmuştur. Diğer yandan, en yüksek ABTS (2351 mg TE/100 g ka) ve DPPH (475.19 mg TE/100 g ka) antioksidan kapasite değerleri mürver çiçeği infüzyonu ile elde edilmiştir. Gastrointestinal sindirim sonuçlarına göre, infüzyonlarda bulunan fenoliklerin elde edilme oranının ağızdan bağırsağa değişken olduğu bulunmuştur. Çalışmada elde edilen sonuçlara göre, bu bitkilerin yüksek besinsel içerikleri nedeniyle değerli bir içecek alternatifi olduğu sonucuna varılabilmektedir.

Anahtar Kelimeler: Bitkisel infüzyonlar, Antioksidan kapasite, Biyoerişilebilirlik, Fenolik bileşikler

Abbreviations: GAE, gallic acid equivalents; TE, Trolox equivalents; Trolox, 6-hydroxy-2,5,7,8 tetramethyl chroman-2-carboxylic acid; CE, catechin equivalents; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2 azinobis (3 ethlybenzothiazoline-6-sulfonic acid) diammonuium salt); TPTZ, Triphenyltetrazolium; CUPRAC, cupric ion reducing antioxidant capacity; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; TRAP, total radical-trapping antioxidant parameter; dm, dry matter.

1. INTRODUCTION

A great number of fruits, vegetables, aromatic, spicy, medicinal and other plants are natural sources of phytochemicals including phenolic compounds, nitrogen compounds, vitamins, terpenoids and other endogenous metabolites (Kamiloglu et al., 2014). Epidemiological studies have shown that many of the phytochemicals possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities (Owen et al., 2000; Zielinski et al., 2014). They are also associated with reduced risks of cancer, cardiovascular disease, diabetes and lower mortality rates of several human diseases (Sun et al., 2002). Therefore, there has been a great interest on the bioactivity phenolic compounds present in the herb extracts as well as infusion/tea prepared by these herbs (Ozkan et al., 2016; Ozkan et al., 2021).

The response of antioxidants may differ according to radical or oxidant sources; therefore, multi assays such as ORAC assay, total TRAP assay, β-carotene or crocin-bleaching assay, total phenol assay by using the Folin-Ciocalteu reagent, ABTS assay, DPPH radical scavenging capacity assay, FRAP assay, and CUPRAC assay are prefered to reflect the

mechanism of action of all radical sources or all antioxidants in a complex system (Karadag et al., 2009).

In the recent years, more attention has been given to the studies about bioaccessibility and bioavailability of polyphenols to predict the release of these phytochemicals from the food matrix and absorption of their metabolites in the throughout the gut epithelium (Kamiloglu et al., 2017; Ozkan et al., 2021, 2022a, 2022b; Wu et al., 2017). In order to estimate the bioaccessibility and bioavailability, several in vitro digestion methods, including static (Minekus et al., 2014) and dynamic (Menard et al., 2014) methods, have been proposed as an alternative to *in vivo* methods due to their lower cost, time and energy saving properties and their independence from ethical concerns and physiological factors (Tang et al., 2006). Minekus et al. (2014) proposed a standardized and practical static digestion model due to the diversity of in vitro digestion model conditions has limited the ability to compare results across different studies.

Due to the strong relationship between benefial effects of the phenolic compounds and their bioaccessibilities/bioavailiabilities, it is a necessity to conduct in vitro digestion study in order to predict their metabolic fates and final bioactivities. In the study described here, the herbs were selected owing to their widely consumption and health promoting effects. Therefore, the objective of the present study was to evaluate the phenolic contents and the antioxidant potentials of the herbal infusions prepared by *Aspalathus linearis* (rooibos leaves), *Plantago lanceolata* (narrow leaf plantain), *Sambucus nigra* (elderberry flowers), *Rhamnus frangula* (alder buckthorn bark) and *Equisetum arvense* (field horsetail leaves). In addition to these, the retention of phenolics and their antioxidant capacities in herbal infusions were evaluated after in vitro gastrointestinal digestion.

2. MATERIALS and METHODS

2.1. Plant Materials

Herbs including *Aspalathus linearis* (rooibos), *Plantago lanceolata* (narrow leaf plantain), *Sambucus nigra* (elderberry), *Rhamnus frangula* (alder buckthorn) and *Equisetum arvense* (field horsetail) were obtained as three independent biological replicates from Arifoğlu brand in Istanbul, Türkiye.

2.2. Chemicals

For simulated in vitro digestion, α-amylase (EC 3.2.1.1, from human saliva), pepsin (EC 3.4.23.1, from porcine gastric mucosa), pancreatin (EC 232.468.9, from porcine pancreas, contains trypsin, amylase and lipase) and bile were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

The Folin–Ciocalteu reagent, gallic acid, catechin, neocuproine, DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8 tetramethyl chroman-2-carboxylic acid), ABTS (2,2 azinobis (3 ethlybenzothiazoline-6-sulfonic acid) diammonuium salt), TPTZ (Triphenyltetrazolium) were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). All other reagents used were of analytical grade.

2.3. Preparation of the Infusion

Herbal infusions were prepared according to ISO Standard of "Preparation of a liquor of tea for use in sensory tests". For this purpose, 2 grams of herb was weighed and placed into a beaker and mixed with 100 mL of boiling water. Then, it was allowed to brew for 6 minutes. After, each infusion was filtered and left for cooling (BSI, 1980).

2.4. In Vitro Gastrointestinal Digestion Model

The in vitro gastrointestinal digestion protocol was conducted based on Minekus et al. (2014). This method comprises sequentially simulated oral, gastric and intestinal digestion steps.

The oral digestion was simulated by mixing 5 mL of each herbal infusion with 3.5 mL of salivary juice, 0.5 mL of α-amylase solution (25 µkat/mL), 25 µL of 0.3 mol/L $CaCl_2$, and 0.975 µL of distilled water to obtained a final volume of 10 mL. This mixture was incubated at 37 °C in a shaking water bath (Memmert SV 1422, Memmert GmbH & Co. Nürnberg, Germany) for 2 min. After simulated oral digestion, 2 mL samples were collected for each infusion.

The gastric digestion was simulated by adding 8 mL of gastric juice, 1.28 mL of pepsin solution (417 µkat/mL), 4 µL of 0.3 mol/L $CaCl_2$ into the remaining oral bolus and the pH was adjusted to 3.0 using 1 mol/L HCl. The total volume of this mixture was adjusted to 16 mL using distilled water. Then, the mixture was incubated in a shaking water bath at 37 °C for 2 h. After simulated gastric digestion, 2 mL aliquots were seperated from each infusion.

The intestinal digestion was simulated bymixing the gastric chyme with 7.7 mL of intestinal juice, 3.5 mL of pancreatin (13 µkat/mL), 1.75 mL 160 mmol/L bile and 28 µL 0.3

mol/L $CaCl_2$. The pH of the mixture was adjusted to 7.0 using 1 mol/L NaOH. The total volume of this mixture was completed to 28 mL using distilled water. The mixture was incubated in a shaking water bath at 37 °C for 2 h.

A blank (use of same amount of water instead of samples) was also incubated under the same conditions in order to correct the any interference from the simulated digestive fluids.

All samples obtained from the simulated oral, gastric and intestinal digestion steps were centrifuged (Hettich, Tuttlingen, Germany) at 23000 g and 4 °C for 5 min. Supernatants were kept at -20 °C until further analysis.

2.5. Determination of Total Phenolics and Antioxidant Capacity

Total phenolic content was analyzed using Folin – Ciocalteu reagent (Singleton and Rossi, 1965). The results are showed as mg gallic acid equivalents (GAE) per 100 g dw sample (Singleton & Rossi, 1965).

Total flavonoid content assay was conducted based on Dewanto et al. (2002). The results are stated as mg catechin equivalents (CE) per 100 g dw sample (Dewanto et al., 2002).

Total antioxidant capacity of the samples was determined by FRAP, CUPRAC, ABTS and DPPH assays (Apak et al., 2004; Benzie & Strain, 1996; Kumaran & Joel Karunakaran, 2006; Miller & Rice-Evans, 1997). In all protocols, the results are given as mg Trolox equivalents (TE) per 100 g dw of sample.

2.6. Statistical Analysis

All analyses were performed at least in three replicates. Results were stated as mean ± standard deviation. Statistical analysis was carried out using SPSS software (version 20.0, SPSS Inc. Chicago, IL, USA). Results were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test ($p < 0.05$). The correlation coefficients (R) were calculated using Microsoft Office Excel 2011 software (Microsoft Co. Redmond, WA, USA).

3. RESULTS and DISCUSSION

3.1. Recovery of Phenolic Compounds During In Vitro Gastrointestinal Digestion

The effect of in vitro digestion on total phenolic and total flavonoid content of herbal infusions was shown in Table 1.

Table 1. Changes in the total phenolic and total flavonoid contents of herbal infusions during simulated in vitro digestion[a]

Sample	Undigested	Simulated oral digestion	Simulated gastric digestion	Simulated intestinal digestion
Total phenolic content (mg GAE/100 g dw)				
Equisetum arvense (field horsetail)	781.60 ± 37^{cC}	1074.75 ± 13^{bC}	737.81 ± 48^{cE}	4313.73 ± 39^{aC}
Rhamnu sfrangula (alder buckthorn)	1198.60 ± 57^{dB}	1753.72 ± 94^{cB}	2614.77 ± 10^{bB}	4012.77 ± 30^{aD}
Sambucu snigra (elderberry)	1890.88 ± 67^{dA}	2268.72 ± 19^{cA}	3314.07 ± 35^{bA}	6646.55 ± 40^{aA}
Aspalathus linearis (rooibos)	2191.18 ± 93^{bA}	2343.19 ± 21^{bA}	2097.89 ± 21^{bC}	5875.84 ± 55^{aB}
Plantago lanceolata (narrow leaf plantain)	783.70 ± 79^{cC}	787.35 ± 74^{cC}	1230.36 ± 14^{bD}	4229.33 ± 38^{aCD}
Total flavonoid content (mg CE/100 g dw)				
Equisetum arvense (field horsetail)	342.14 ± 24^{aC}	373.82 ± 52^{aC}	226.80 ± 45^{aD}	243.22 ± 24^{aC}
Rhamnu sfrangula (alder buckthorn)	838.05 ± 63^{bB}	1280.43 ± 67^{aB}	554.41 ± 10^{cC}	1289.65 ± 24^{aAB}
Sambucus nigra (elderberry)	1163.40 ± 77^{cAB}	1917.54 ± 19^{aA}	1972.82 ± 20^{aA}	1408.43 ± 13^{bA}
Aspalathu slinearis (rooibos)	1226.14 ± 93^{bA}	1669.15 ± 20^{aA}	1144.81 ± 13^{bB}	1204.80 ± 36^{bB}
Plantago lanceolata (narrow leaf plantain)	357.31 ± 19^{aC}	396.17 ± 47^{aC}	350.40 ± 39^{aD}	67.88 ± 24^{bD}

[a] The data presented in this table consist of average values ± standard deviation of three independent batches. Total phenolic and total flavonoid content is expressed as mg of gallic acid equivalents (GAE), and catechin equivalents (CE) per 100 g of dry weight (dw) of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

The difference in total phenolic and total flavonoid content was found to be statistically significant ($p < 0.05$) for undigested, simulated oral digested, simulated gastric digested and simulated intestinal digested samples. For undigested samples, the highest total phenolic and total flavonoid value were obtained with *Aspalathus linearis* as 2191.18 ± 93 mg GAE/100g dw and 1226.14±93 mg CE/100 g dw, respectively.

Total phenolic content of each herbal infusion used in this study was found to be higher than those of *Zingiber officinale* (Ginger; 710±90 mg GAE/100g dw), *Chamomillae romanae* (Daisy; 730±170 mg GAE/100g dw) and *Tilia platyphyllos* (Linden; 860±70 mg GAE/100g dw) which are the most widely known infusions throughout the world. Total phenolic content of those infusons were reported as 710±90, 730±170, 860±70 mg GAE/100g dry sample, respectively (Toydemir et al., 2015). On the other hand, it has been reported that brewing time is an effective factor on the total phenolic content of the infusions. For instance; previous studies reported that the total phenolic content of *Aspalathus linearis* was found to be 2350±246 mg GAE/100g dw with 5 min (Joubert & de Beer, 2012), while 3750±235 mg GAE/100g dw was found with an hour brewing duration (Chan et al., 2010).

After simulated oral digestion, total phenolic content of *Aspalathus linearis* and *Plantago lanceolate*; total flavonoid content of *Equisetum arvense* and *Plantago lanceolata* did not change significantly ($p > 0.05$), whereas the results of other herbal infusions were obtained significantly higher compared to undigested samples ($p < 0.05$). After the simulated gastric digestion, there was a significant increase in only total phenolic content of *Rhamnu sfrangula*, *Sambucu snigra* and *Plantago lanceolate* ($p < 0.05$); while there were no any increase in total flavonoid content of all herbal infusions. After simulated intestinal digestion further significant increases in total phenolic content were determined ($p < 0.05$). On the other hand, total flavonoid content remained stable with the exception of *Rhamnu sfrangula*, *Sambucus nigra* and *Plantago lanceolate*.

Correlations between total phenolic and total flavonoid content assays were differ depending on whether the samples is undigested or digested as well as the stage of digestion. The highest correlation coefficient was found to be R = 0.95 for simulated oral digested samples, followed by undigested samples, simulated gastric digested samples and simulated intestinal digested samples with 0.94, 0.70 and 0.37 correlation coefficients, respectively. In the study of Pirbalouti et al. (2013), correlation coefficient between total phenolic and total flavonoid contents was determined as 0.954 which is highly compatible with the correlation coefficient of simulated oral digested samples and undigested samples in the present experiment. It can be concluded from the results that infusions with high levels of phenolic content were also rich in flavonoids (Oh et al., 2013).

3.2. Antioxidant Capacity of Digested Polyphenols

The effect of in vitro digestion on total antioxidant capacity of herbal infusions was shown in Table 2.

The variation in total antioxidant capacity of infusions was found to be statistically significant ($p < 0.05$) for undigested, simulated oral digested, simulated gastric digested and simulated intestinel digestinal digested samples. For undigested samples, the highest total antioxidant capacity values were obtained with *Sambucus nigra* and *Aspalathus linearis*. In a study of Wong et al. (2015), total antioxidant capacity of rooibos herbal infusion was found to be 2496 mg TE/100 g dw by FRAP assay which is similar to the that of present study as 2634.95 mg TE/100 g dw. On the other hand, according to Oh et al. (2013), total antioxidant capacity of rooibos by using ABTS assay was found to be 3931 mg AAE/100 g dw (AAE; ascorbic acid equivalent) which was higher than that of the present study as 2342.91 mg TE/100 g dw,

probably due to the difference in the standard compound. It has been indicated that the variety of standards affect the chemical properties including redox potential, stoichiometries, kinetics, solubility and auto-oxidation susceptibility (Poljšak & Raspor, 2008).

Table 2. Changes in the total antioxidant capacity of herbal infusions during simulated in vitro digestion[a]

Sample	Undigested	Simulated oral digestion	Simulated gastric digestion	Simulated intestinal digestion
2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS)				
Equisetum arvense (field horsetail)	1884.31±15^{cAB}	1129.56±0.02^{dE}	2508.41±0.01^{bD}	3165.49±0.15^{aD}
Rhamnu sfrangula (alder buckthorn)	2114.73±12^{cAB}	2091.36±0.02^{cC}	3217.22±0.001^{bC}	3460.93±0.27^{aC}
Sambucu snigra (elderberry)	2351±11^{cA}	3057.23±0.03^{cB}	5579.93±0.22^{bA}	8584.64±0.32^{aA}
Aspalathus linearis (rooibos)	2342.91±11^{dAB}	3612.84±0.03^{cA}	4285.69±0.23^{bB}	7854.56±0.38^{aB}
Plantago lanceolata (narrow leaf plantain)	1287.67±12^{dB}	1361.86±0.03^{cD}	2434.90±0.11^{bE}	3035.56±0.19^{aE}
Cupric ion reducing antioxidant capacity (CUPRAC)				
Equisetum arvense (field horsetail)	1100.12±11^{dB}	1914.72±0.15^{cE}	2027.37±0.02^{bE}	4425.04±0.6^{aD}
Rhamnu sfrangula (alder buckthorn)	1189.35±113^{dB}	2542.64±0.16^{cC}	3250.79±0.15^{bD}	5495.99±0.8^{aC}
Sambucus nigra (elderberry)	2981.28±155^{dA}	14209.30±0.36^{cA}	15714.67±0.23^{bA}	16664.53±0.5^{aA}
Aspalathus slinearis (rooibos)	3124.12±135^{dA}	3883.72±0.37^{cB}	5048.47±0.17^{bB}	6743.48±0.11^{aB}
Plantago lanceolata (narrow leaf plantain)	1104.96±112^{dC}	2405.68±0.24^{cD}	3255.78±0.20^{bC}	4154.36±0.56^{aE}
2,2-diphenyl-1-picrylhydrazyl (DPPH)				
Equisetum arvense (field horsetail)	447.14±14^{cAB}	1204.37±0.04^{aD}	1221.29±0.16^{aC}	690.39±0.17^{bC}
Rhamnu sfrangula (alder buckthorn)	240.42±23^{dB}	797.88±0.3^{bE}	1180.08±0.7^{aD}	690±0.25^{cC}
Sambucus nigra (elderberry)	475.19±52^{dA}	1546.03±0.4^{cA}	3515.23±0.9^{aA}	1968.12±0.03^{bA}
Aspalathus slinearis (rooibos)	439.48±39^{dAB}	1395.17±0.01^{cB}	2588.33±0.11^{aB}	1810.12±0.7^{bB}
Plantago lanceolata (narrow leaf plantain)	413.60±56^{cAB}	1231.52±0.01^{aC}	1090.04±0.9^{aE}	632±0.03^{bD}
Ferric reducing antioxidant power (FRAP)				
Equisetum arvense (field horsetail)	626.01±57^{aD}	417.89±0.03^{cE}	308.79±0.01^{dE}	546.01±0.51^{bE}
Rhamnu sfrangula (alder buckthorn)	625.26±55^{bD}	542.20±0.05^{bC}	587.39±0.3^{bD}	855.37±0.31^{aC}
Sambucus nigra (elderberry)	2309.15±12^{aB}	2190.53±0.02^{bA}	2169.76±0.16^{bA}	1503.47±0.33^{cA}
Aspalathus slinearis (rooibos)	2634.95±13^{aA}	1746.99±0.16^{bB}	1663.09±0.7^{cB}	727±0.62^{dD}
Plantago lanceolata (narrow leaf plantain)	836.56±58^{aC}	512.48±0.01^{bD}	609.38±0.3^{bC}	883.97±0.22^{aB}

[a] The data presented in this table consist of average values ± standard deviation of three independent batches. Total antioxidant capacity is expressed as mg Trolox equivalents (TE) per 100 g of dw of sample. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

After simulated oral digestion, total antioxidant capacity values of herbal infusions changed significantly ($p < 0.05$). After simulated gastric digestion, significant increases in total antioxidant capacity (in case of ABTS and CUPRAC) were obtained in comparison with the results obtained after simulated oral digestion ($p < 0.05$). After simulated intestinal digestion, further significant increases in total antioxidant capacity (in case of ABTS and CUPRAC) were obtained ($p < 0.05$). On the other hand, total antioxidant capacity measured by DPPH assay showed a significant decrease in comparison with the results obtained after simulated gastric digestion ($p < 0.05$).

Due to the fact that the radical scavenging capacity of phenolic compounds many depend on pH of the environment, the antioxidant potential may be variable during digestion. Besides, there may be structural alterations in the phenolic substances by ionizing the hydroxyl groups due to passing from gastric to intestinal conditions, resulting in improved antioxidant capacity at higher pH values. Similarly, Tagliazucchi et al. (2010) indicated that the free radical scavenging activities of some phenolic compounds such as gallic and caffeic acids, catechin, quercetin and resveratrol were enhanced after intestinal digestion.

Furthermore, it has been recommended that antioxidant potential of food products could be assessed by using multiple assays because of their complex multiphase structures that may have an impact on the results (Apak et al., 2016). Thereby, correlations between total antioxidant capacity assays were also investigated. For both the undigested and digested samples, from moderate to high correlations were determined; the highest (R = 0.6938-0.9874) correlation was indicated between FRAP and CUPRAC assays among others. Similarly, Kamiloglu et al. (2017) found the highest correlations between FRAP and CUPRAC assays changing between 0.7492 and 0.9704 (Kamiloglu et al., 2014). It can also be deduced from the results that ABTS and DPPH assays could be more appropriate for evaluating total antioxidant capacity after simulated gastric digestion in accordance with the pH conditions.

4. CONCLUSION

The present study focused on the evaluation of total phenolics, total flavonoids, total antioxidant capacities as well as examination of digestive stability and bioaccessibility of the phenolics in herbal infusions using the standardized static in vitro digestion protocol. It was found that these infusions can be consumed as an alternative to most known herbal teas with compatible phenolic and flavonoid contents. Besides, total phenolic content and total antioxidant capacity increased significantly as a result of digestion, whereas the loss of total flavonoid content of

some of the infusions were obtained. Moreover, it is clear that the measurement of antioxidant capacity of food products cannot be evaluated satisfactorily using a single antioxidant assay due to their complex multiphase systems, which can influence the results. Therefore, more than one assay with different mechanisms should be applied to evaluate total antioxidant capacities of food products. As a future aspect, it can be suggested that conducting in vitro Caco-2 cell culture method to assess the final absorption of phenolic compounds may be helpful to evaluate the fate of the infusion phenolics in human digestive system.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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Characterization of Anatolian Bee Breads by Principal Component Analysis Based on Their Physicochemical and Chemical Characteristics

Anadolu Arı Ekmeklerinin Fizikokimyasal ve Kimyasal Özelliklerine Dayalı Temel Bileşen Analizi ile Karakterizasyonu

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Abstract

Bee bread is a fermented bee product which is the mixture of pollen and honey. This substance is actually the main source of food for honey-bee workers. Due to its rich therapeutic properties, bee bread has gained an increasing interest for human consumption in recent years. The purpose of this study was to compare the physicochemical characteristics and chemical composition of bee bread samples collected from seven different regions in Anatolia. Various measurements such as pH, electrical conductivity, colour, colour intensity, and optical density were performed. Together with the mentioned, the content of moisture, ash, total protein, and free acidity were analyzed. Principal component analysis (PCA) was applied to analyze results in order to classify the bee breads from different regions. This study contributes to the chemical and physicochemical knowledge of this scarcely explored natural bee-product.

Keywords: Bee bread, Perga, Total protein content, Colour analysis, Free acidity

Özet

Arı ekmeği, polen ve bal karışımı olan fermente bir arı ürünüdür. Bu madde aslında bal arısı işçilerinin ana besin kaynağıdır. Arı ekmeği, zengin tedavi edici özelliklerinden dolayı son yıllarda insan tüketiminde artan bir ilgi görmektedir. Bu çalışmanın amacı, Anadolu'nun yedi farklı bölgesinden toplanan arı ekmeği örneklerinin fizikokimyasal özelliklerini ve kimyasal bileşimini karşılaştırmaktır. pH, elektriksel iletkenlik, renk, renk yoğunluğu ve optik yoğunluk gibi çeşitli ölçümler yapılmıştır. Bunlarla birlikte nem, kül, toplam protein ve serbest asit içeriği analiz edildi. Farklı bölgelerden arı ekmeklerini sınıflandırmak için sonuçları analiz etmek için temel bileşen analizi (PCA) uygulanmıştır. Bu çalışma, nadiren keşfedilen bu doğal arı ürününün kimyasal ve fizikokimyasal bilgisine katkıda bulunmaktadır.

Anahtar Kelimeler: Arı ekmeği, Perga, Total protein miktarı, Renk analizi, Serbest asitlik
Abbreviations: PCA, Principal component analysis; OD, Optical density, ANOVA, Analysis of variance

1. INTRODUCTION

Honey, bee-pollen, propolis, royal jelly, and bee venom are among the most popular bee products which have been used in traditional medicine. These products have been known to increase body resistance due to their bioactive ingredients (Kolaylı & Keskin, 2020). In the recent years, scientific researches on the therapeutic effects, different bioactivities, physicochemical characteristics, and chemical compositions of bee products have been increased on a large scale (Alvarez-Suarez, 2017; Bogdanov, 2011; Kaygusuz et al., 2016; Nainu et al., 2021; Tezcan et al., 2011).

Bee bread (perga) is also a unique bee product which is poorly known until a few years ago due to the difficulties in collecting (Bakour et al., 2022). However, the appropriate collecting methods that beekeepers could perform without destroying the hive were improved. Thus, this product have been provided to human consumption. Bee bread is produced by the older female adult bees mixing pollen grains with nectar, honey, and other bee secretions such as digestive enzymes in their saliva. The mixture is subjected to lactic acid fermentation and becomes bee bread in about two weeks. As a fermented product, it is kept with a thin layer of honey and bee wax (Bakour et al., 2022).

The therapeutic properties of bee bread samples such as antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic activities were reported in the literature (Bakour et al., 2022; Mohammad et al., 2020; Nagai et al., 2004; Urcan et al., 2017; Pełka et al., 2021). The chemical compositions of bee bread from worldwide were also studied by many authors (Adaškevičiūtė et al., 2019; Bakour et al., 2019; Dranca et al., 2020; Ivanišová et al. 2015; Mohammad et al., 2020; Sobral et al., 2017; Urcan et al., 2018; Zuluaga et al., 2015). According to these studies, bee bread mainly composed of water, protein, carbohydrates, fatty acids, organic acids, vitamins, minerals, and several bioactive compounds.

Anatolia is pretty rich with regard to endemic vegetation due to its geographical location. Different bee products such as honey, bee-pollen, and bee venom from Anatolia have been evaluated by our group before (Aftab et al., 2021; Akay et al., 2021; Kalaycıoğlu et al., 2017; Kalaycıoğlu et al., 2021). High antioxidant activities and nutritional contents such as minerals, vitamins, sugar, and organic contents were reported in these studies. Anatolian bee-

bread was evaluated by several groups (Bayram et al., 2021; Beykaya et al., 2021; Kaplan et al., 2016). However, studies on bee bread seem to be limited when compared with other bee products. It is shown that both the composition of bee bread and the amounts of the compounds varies depending on the region, melliferous plants and climatic conditions of that region, and collecting season (Andjelkovic et al., 2012; Baltrušaitytė et al., 2007). Bayram et al. (2021) and Beykaya et al. (2021) used chemometric techniques in order to show the discrimination between bee breads.

The objective of the present work was to determine some physicochemical properties such as pH, electrical conductivity, colour analysis, colour intensity, and optical density of bee bread samples collected from seven different regions in Anatolia. The chemical analysis such as moisture, ash, total protein contents and free acidity were also determined. Moreover, the data were evaluated by principal component analysis to discriminate the bee bread samples according to their origins.

2. MATERIALS and METHODS

2.1. Bee Bread Samples and the Sample Preparation for the Analysis

Bee bread samples were collected from different locations such as Bingöl, Kırşehir, Ankara, Trabzon, Zonguldak, Erzurum and Niğde during 2021 (Figure 1). All samples were stored at 4°C until further analysis.

Figure 1. Location map of sample sites

Bee bread samples were finely grounded in a laboratory mortar. Fifty hundred mg of powdered samples were accurately weighed. Each sample was extracted with 10 mL of

deionized water by vortexing for 5 min at 3000 rpm and sonicating for 15 min. The mixture was centrifuged at 3500 rpm for 15 min. The supernatant was filtered through 0.45 µm pore-sized micro filters before analysis. These extracts were used for the measurement of pH, colour intensity, colour analysis, optical density, electrical conductivity and the determination of total protein content and free acidity.

2.2. Chemicals

Sodium hydroxide (NaOH), sodium chloride (NaCl), potassium chloride (KCl), Coomassie Brilliant Blue G-250, ethanol, phosphoric acid (H_3PO_4), bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany).

2.3. Physicochemical Analysis of Bee Bread Samples

2.3.1. Determination of pH and Electrical Conductivity

A pH meter (Thermo Scientific, USA) was used to measure the pH values of the bee bread samples.

Electrical conductivity of the samples was measured using a conductivity meter (Wissenschaftl Techn, Weilheim, Germany). The instrument was calibrated using 0.01 M KCl. The results were reported as mS/cm. The electrical conductivity of the deionized water was determined below 5 mS/cm.

2.3.2. Colour Analysis, Colour Intensity, and Optical Density

The colour analysis of the bee bread samples was measured according to Pfund colour scale which the widely used in the colour measurement of honey. Briefly, the absorbance value of the samples was meaured at 635 nm using a Shimadzu 1800 UV-VIS spectrophotometer (Japan). Pfund values of the samples were calculated according to the following equation (Sant'ana et al., 2013) (Equation 1).

$$mm\ Pfund = -38.70 + (371.39xA) \text{ (Equation 1)}$$

mm Pfund: Colour intensity in Pfund scale

A: Absorbance value of bee bread sample

The colour intensity was measured using the method of Beretta et al. (2005). The absorbance of bee bread extracts was measured at 450 nm and 720 nm using a spectrophotometer. The difference between the absorbance at 450 nm and 720 nm was expressed as mAU.

For the determination of optical density (OD), the absorbance of each bee bread sample extract was measured at 530 nm against deionized water using a spectrophotometer (Wakhle, 1997).

2.4. Chemical Analysis of Bee Bread Samples

2.4.1. Moisture Content

The moisture contents of the bee bread samples were measured by the loss on drying technique using a moisture analyzer (Shimadzu, Japan). The samples were weighed on moisture balance and superheated until the end of the drying period by infrared. The temperature was set at 105 °C. All the samples reached equilibrium at 10 min. The results were recorded as g/100g moisture.

2.4.2. Ash Content

Ash content of the bee bread samples was determined according to the methods of AOAC (1999). Each sample was accurately weighed as 0.2000 g and was placed in crucibles. Then, the samples were incinerated at 550 °C in a burning muffle for 3 h. After cooling at room temperature, the obtained ash was weighed. Ash percentage (g ash/100 g bee bread) of the samples were calculated according to the Equation 2:

$$\%Ash = \frac{m_{ash} x 100}{m_{sample}} \text{ (Equation 2)}$$

2.4.3. Total Protein Content

The total protein content of the bee bread samples was analyzed by the Bradford method with a little modification (Bradford, 1976). Twenty mg of Coomassie Brilliant Blue G-250 was dissolved in 10 mL 95% ethanol solution (95% ethanol-5% water, v/v). Then, 20 mL 85% H_3PO_4 solution (85% H_3PO_4 – 15% water, v/v) were added and the resulting solution was diluted to a final volume of 100 mL. Fifty hundred µL of each bee bread sample extract was added to 5 mL of this solution. The Coomassie Brilliant Blue forms a protein-dye complex. After 5 minutes of incubation, absorbance was measured at 595 nm against an albumin standard solution of bovine serum (10-100 µg/0.1 mL) in 0.15 M NaCl.

2.4.4. Determination of Free Acidity

Free acidity was determined by potentiometric titration. The solution was titrated with 0.05 mol/L NaOH to pH 8.30. The results were expressed in miliequivalent of acid per kg of bee bread.

2.5. Statistical Analysis

All the analysis was done three replicates. The results were given as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was applied to evaluate the significance of differences in the obtained mean values of the bee bread samples at $p \leq 0.05$. Principal component analysis (PCA) was also used to visualize the differences and similarities among the bee bread samples. All the statistical calculations were performed using Minitab 16 statistical software program for Windows.

3. RESULTS and DISCUSSION

3.1. Physicochemical Characterization of Bee Bread

The physicochemical parameters of the bee bread samples were presented in Table 1. As it is seen from the table, all the samples were acidic (pH: 3.66-4.16) that insures their freshness. Among the samples, the bee bread collected from Trabzon was the most acidic (pH: 3.66) followed by Zonguldak (pH: 3.84). The lowest acidity was detected in bee bread collected from Bingöl (pH: 4.16). Analysis of variances revealed that the values of pH of the tested bee breads were not identified as statistically different ($p > 0.05$). The pH values found in this study are comparatively in line with past reports for bee bread pH value: 4.04 in a Romanian bee bread (Dranca et. Al., 2020) and between 4.11- 4.44 in Lithuanian bee breads (2.93–4.08) (Adaškevičiūtė et al., 2019).

The electrical conductivity (EC) of all studied bee bread samples ranged from 4.63 to 5.64 mS/cm. The highest EC value was determined in bee bread samples from Trabzon (5.64 mS/cm) whereas the lowest EC value was recorded for Ankara (4.63 mS/cm). Performed ANOVA revealed that these differences were statistically significant at $p \leq 0.05$.

The colour of bee bread samples were evaluated using Pfund scale, colour intensity, and optical density. The highest Pfund value belonged to Zonguldak bee bread with 109.8 mm whereas the lowest Pfund value was registered with Trabzon bee bread (61.58 mm). The other samples were changed between 65.28 mm and 95.00 mm. USDA-approved colour standards (1985) reported that the samples having Pfund scale between 50 mm and 85 mm are light amber in colour whereas between 85 mm-114 mm value shows amber (USDA, 1985). According to this standard, bee breads from Zonguldak and Erzurum were amber in colour whereas all the other samples were light amber. There is a significant difference in colour between all studied types of bee bread ($p \leq 0.05$).

Colour intensities of the bee bread samples were represented by the AB450. AB450 values ranged from 94.03 to 194.6 mAU (Table 1). The results showed that there is a significant difference between studied types of bee bread in colour intensity ($p \leq 0.05$). Zonguldak bee bread, which showed the highest Pfund value, also presented the highest colour intensity (194.6 mAU) followed by Erzurum (129.6) and Bingöl (107.6 mAU) bee breads while Trabzon showed the lowest colour intensity (92.66 mAU). The correlation between colour and colour intensity was found to be as 0.89.

The optical density which is a means of colour classification were changed between the 0.27 (Trabzon bee bread) and 0.40 (Zonguldak bee bread) mAU. A statistically significant difference was obtained for the optical density of the samples ($p \leq 0.05$). The correlation between colour and colour intensity was found to be as 0.86.

Table 1. Physicochemical characteristics of bee bread samples

Bee bread	pH	EC (mS/cm)	Colour (Pfund scale, mm)	Colour intensity (mAU)	Optical density (mAU)
Ankara	3.91 ± 0.03	4.63 ± 0.02	65.28 ± 1.12	94.03 ± 4.16	0.32 ± 0.03
Bingöl	4.16 ± 0.05	5.06 ± 0.03	80.15 ± 2.63	107.6 ± 4.16	0.36 ± 0.01
Erzurum	4.03 ± 0.04	5.02 ± 0.04	95.00 ± 3.09	129.6 ± 4.6	0.38 ± 0.02
Kırşehir	3.84 ± 0.01	5.01 ± 0.04	69.00 ± 1.65	97.6 ± 2.3	0.29 ± 0.01
Niğde	3.92 ± 0.02	5.40 ± 0.05	69.00 ± 2.10	98.2 ± 3.4	0.29 ± 0.01
Trabzon	3.66 ± 0.02	5.64 ± 0.03	61.58 ± 2.18	92.66 ± 4.1	0.27 ± 0.01
Zonguldak	3.80 ± 0.01	5.10 ± 0.04	109.8 ± 3.9	194.6 ± 2.4	0.40 ± 0.04

Each of the physicochemical characteristics mentioned in this study were reported for Anatolian bee breads for the first time.

3.2. Chemical Characterization of Bee Bread

Table 2 displays the moisture, ash, total protein content and free acidity in bee bread samples. Moisture content is closely related with quality and stability of bee products. Bee bread is known as a hygroscopic material which is highly reactive to fungal or microbial molecules. In order to avoid this, bee bread is generally stored in dried or frozen form. The moisture of bee bread samples at 105°C was found to be between 11.54-23.07 g/100g. Bee bread collected from

Trabzon showed the highest moisture content (23.0 g/100g) whereas Kırşehir bee bread presented the lowest moisture content (11.54 g/100g). Performed ANOVA revealed that these differences were statistically significant at $p \leq 0.05$. The results obtained in this study were in consistent with the other bee bread samples from Anatolia. Beykaya et al. (2021) reported the moisture content of 10 bee bread sample between 10.13-18.10 g/100g. In the study of Kaplan et al. (2016), 8 Anatolian bee bread samples showed different moisture contents changing between 11.41 and 15.89 g/100g.

Table 2. Chemical characteristics of bee bread samples

Bee bread	Moisture (g/100g)	Ash (g/100g)	Total protein (g/100g)	Free acidity (mEq/kg)
Ankara	19.60 ± 0.05	1.97 ± 0.01	20.16 ± 1.12	531 ± 17
Bingöl	17.54 ± 0.02	2.93 ± 0.02	22.18 ± 2.63	493 ± 12
Erzurum	20.00 ± 0.09	2.78 ± 0.04	23.85 ± 3.09	518 ± 16
Kırşehir	11.54 ± 0.01	2.82 ± 0.03	23.09 ± 1.65	548 ± 18
Niğde	12.96 ± 0.03	2.85 ± 0.05	24.02 ± 2.10	527 ± 15
Trabzon	23.07 ± 0.10	2.44 ± 0.01	21.67 ± 2.18	569 ± 20
Zonguldak	19.04 ± 0.04	2.39 ± 0.02	22.45 ± 3.9	558 ± 18

The content of ash which attributes to any inorganic materials such as mineral, was between 1.97 (Ankara bee bread)-2.93 (Bingöl bee bread) g/100g. There is no significant difference remarked between samples in ash conttent ($p > 0.05$). The result was in the similar range for bee bread samples from different regions in Anatolia and the world. Kaplan et al. (2016) reported the ash content of Anatolian bee bread samples as 1.93-2.62 g/100g. The mean ash content for Columbian bee bread was reported as 2.45 (Zuluaga et al., 2015) and 3.42% in Romanian bee bread (Dranca et. al., 2020).

Standard protein level in bee pollen is reported as 15 g/100g. The total protein of all bee bread samples was found to be above this standard as seen in Table 2. The highest total protein content belonged to Niğde bee bread (24.02 g/100g) whereas Ankara bee bread had the lowest total protein content (20.16 g/100g). The results were found to be significantly different ($p \leq 0.05$). In the study of Kaplan et al. (2016) total protein contents of Anatolian bee breads

changed between 14.82 and 24.26 g/100g. A Romanian bee bread was reported as including 18.60 g/100g (Dranca et. al., 2020). The mean total protein content of bee bread reported in the literature changed as follows: 19 g/100g in Anatolian bee bread (Beykaya et al., 2021), 19.96 g/100g in Moroccan bee bread (Bakour et al., 2019), 22.26 g/100g in Malaysian bee bread (Mohammad et al., 2020), and 23.1 g/100g in Columbian bee bread (Zuluaga et al., 2015).

Free acidity ranged from 493 meq/kg in bee bread sample from Bingöl and 569 mEq/kg in Trabzon bee bread. These results confirmed the acidic character of the bee bread. It was revealed that the differences in free acidity of bee bread samples were statistically significant at $p \leq 0.05$. Free acidity values found in this study were in consistent with the other studies from literature. The free acidity of a Romanian bee bread was found to be as 543.2 mEq/kg (Dranca et. al., 2020). The mean free acidity of Moroccan bee bread samples was reported as 400 mEq/kg (Bakour et al., 2019).

3.3. PCA Analysis

In order to identify similarities and specificities of bee bread samples analyzed, principal component analysis (PCA) was conducted. Correlated variations with each other was detected whereas the contribution of some variations have lower. As can be seen from Figure 2, except colour intensity, none of the variables are correlated with any principal components (PCs) directly. The negative part of first component (PC1) is related with free acidity, EC, and moisture; the rest of the variables are related with the positive part of PC1.

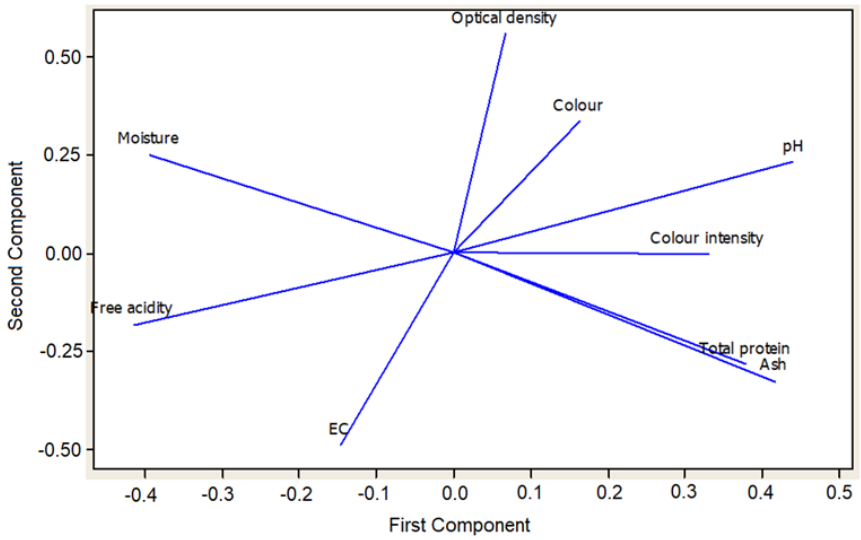

Figure 2. Distribution of the loadings plot for PC1- PC2, generated from a correlation-matrix PCA

The score plot (Figure 3) shows that bee breads separated into four groups. Erzurum and Bingöl bee breads are grouped in the positive part of both PC1 and PC2. Kırşehir and Niğde

bee breads are also close to each other in the positive part of PC1 and negative part of PC2. The samples collected from Ankara and Zonguldak are in negative part of PC1 and positive part of PC2. Only the sample collected from Trabzon is an outlier on the bottom left. The reason for that is significantly lower pH and higher EC values than those of other samples. Therefore, the given variables can be used to distinguish these bee bread samples.

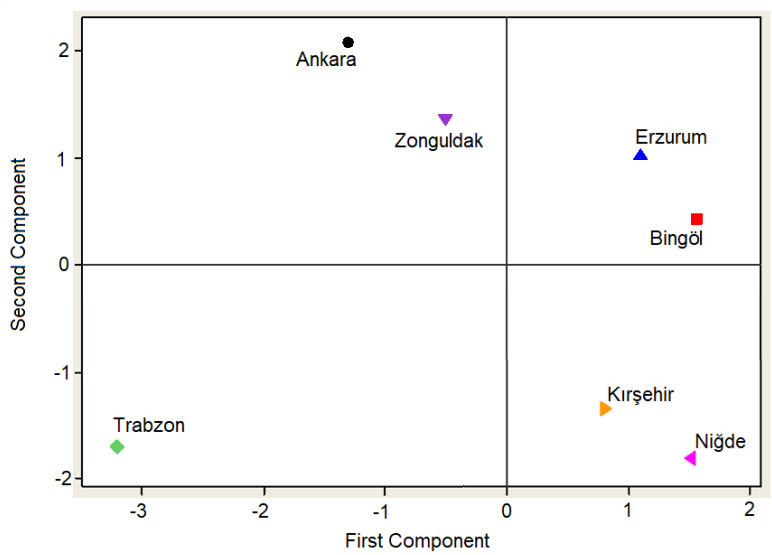

Figure 3. Score plot of bee bread samples on PC1-PC2.

4. CONCLUSION

In recent years, the interest in finding biologically active natural products have been increased with the human population growth. Bee products, which include many components nececssary for basic life functions, are one the important natural products. It is necessary to investigate the chemical properties of these marketed products to contribute to the standardization. However, the scientific studies on bee bread are still scarce. In this study, some physicochemical characteristics and chemical properties of 7 Anatolian bee bread collected from different regions were presented. The results revealed statistically significant differences among the samples in terms of electrical conductivity, colour analysis, moisture, ash, protein content and free acidity. According to the results, bee bread can be preferred as a good source of protein. Moreover, this study shows that PCA appear to be a potential tool for discrimination of bee bread using their physicochemical and chemical profiles tested.

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DECLARATIONS

The author declare that has no conflicts of interest.

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Comparison of Different Methods in the Extraction of Phenolic Compounds from Bay Leaf (Laurus nobilis L.)

Defne Yaprağından (Laurus Nobilis L.) Fenolik Bileşiklerin Ekstraksiyonunda Farklı Yöntemlerin Karşılaştırılması

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Abstract

In this study, the microwave-assisted and enzyme-assisted extraction efficiency were compared to solvent extraction. The extraction efficiencies were evaluated in bay leaf extract in terms of phenolic content and antioxidant capacity. The total phenolic content (mg GAE/g) of the extracts from three different extraction methods as a solvent, enzyme-assisted, and microwave-assisted extraction was found 23.29±0.02, 32.45±0.02, and 30.49±0.02, respectively. The highest value for the total phenolic content was found from the enzyme-assisted extraction. DPPH radical scavenging capacity (%) of the extracts from three different extraction methods was found at 36.91%±0.05, 50.72%±0.27, and 41.51%±0.09, respectively. Like the total phenolic content, the highest value for the DPPH radical scavenging capacity was found from the enzyme-assisted extraction. In addition, total dry matter, total ash, total protein, ascorbic acid, and total chlorophyll content of the bay leaf were analyzed.

Keywords: Enzyme assisted extraction, Microwave assisted extraction, Bay leaf, Phenolic compounds

Özet

Bu çalışmada, mikrodalga destekli ve enzim destekli ekstraksiyon verimliliği çözgen ekstraksiyonu ile karşılaştırılmıştır. Defne yaprağı ekstraktında ekstraksiyon verimleri fenolik içerik ve antioksidan kapasitesi açısından değerlendirilmiştir. Geleneksel ekstraksiyon, enzim destekli ve mikrodalga destekli ekstraksiyon olarak üç farklı ekstraksiyon yönteminden elde edilen ekstraktların toplam fenolik içeriği (mg GAE/g) sırasıyla 23.29±0.02, 32.45±0.02 ve

30.49±0.02 olarak bulunmuştur. Toplam fenolik içerik için en yüksek değer enzim destekli ekstraksiyonda belirlenmiştir. Üç farklı ekstraksiyon yönteminden elde edilen ekstraktların DPPH radikal süpürme aktivitesi (%) sırasıyla %36.91±0.05, %50.72±0.27 ve %41.51±0.09 olarak bulunmuştur. Toplam fenolik içerik gibi, DPPH radikal süpürme aktivitesi için en yüksek değer enzim destekli ekstraksiyonda belirlenmiştir. Ayrıca defne yaprağının toplam kuru madde, toplam kül, toplam protein, askorbik asit ve toplam klorofil içeriği belirlenmiştir.

Anahtar Kelimeler: Enzim destekli ekstraksiyon, Mikrodalga destekli ekstraksiyon, Defne yaprağı, Fenolik bileşikler

1. INTRODUCTION

The bay leaf (*Laurus nobilis* L.), an aromatic herb, is one of the oldest and most widely used spice, widely grown in the Mediterranean region (Sayyah et al., 2003). The bay leaf is rich in bioactive compounds which are secondary metabolites that have a positive effect on health (Simić et al., 2003). The plants are known as rich in phenolic compounds which are one of these secondary metabolites. The phenolic compounds are used in food industry because of their nutritional quality, natural colorant, antioxidant function and organoleptic properties such as color, taste, and odor (Li et al., 2008). However, due to the presence of small amounts of these compounds it is necessary to determine the most efficient extraction method.

The extraction is an important stage for the identification and usage of phenolic compounds. The method used for the extraction of phenolic compounds is usually solvent extraction. Since the conventional method has many disadvantages such as the use of excess solvent and high temperature, long application time, alternative methods are under investigation (Pai et al., 2022). In recent years, several alternative methods have been developed such as enzyme-assisted (Anticona et al., 2020; Boulila et al., 2015), microwave-assisted (Muñiz-Márquez et al., 2018), ultrasound-assisted (Chakraborty et al., 2020), and supercritical fluid (Gan et al., 2020; Zulkafli et al., 2014) extraction methods. In this study, microwave assisted and enzyme-assisted methods' extraction efficiency from bay leaves on phenolic content and antioxidant capacity were compared to solvent extraction.

2. MATERIALS and METHODS

2.1. Materials

The dried bay leaves were purchased from a local market in Izmir-Turkey in October 2019. The dried bay leaves were grounded and stored in glass jars at room temperature. The Pectinex Ultra SP-L enzyme used in enzyme assisted extraction was donated by Novozymes (Novozymes, Denmark). All other chemicals and reagents were of analytical grades.

2.2. The Physicochemical Properties of Dried Bay Leaves

The total dry matter, total ash, and total protein content analyses of dried bay leaves were performed according to the official methods of the Association of Official Analytical Chemists (AOAC, 1990). The ascorbic acid (Bajaj & Kaur, 1981) and chlorophyll content (Vernon, 1960) of bay leaves were determined.

2.3. Extraction of Phenolic Compounds from Dried Bay Leaves

The phenolic compounds were extracted by solvent (conventional) extraction, enzyme assisted extraction and microwave assisted extraction methods. The extraction efficiencies were compared.

2.4. Solvent (Conventional) Extraction (SE)

The extraction of phenolic compounds from bay leaf was carried out by the procedure suggested by Muñiz-Márquez et al. (2018) with some modifications. The solvent extraction was carried out using a bay leaf/ethanol (50%), solid/solvent ratio (1:10, w/v), at 25°C extraction temperature for 24 hours extraction.

2.5. Enzyme Assisted Extraction (EAE)

For the enzymatic pretreatment, a 1 g of dried bay leaf powder was dispersed in 10 mL of pure water. The pH value of the sample was adjusted to 5.5 by 0,1 N HCl for the maximum enzyme activity. The selected extraction parameters were the concentration of the enzyme (8%), incubation temperature (45°C) and reaction time (30 min). Extraction was carried out under constant stirring conditions at 150 rpm continuously. After enzymatic pretreatment, solvent extraction with ethanol (50%) was carried out at a bay leaf/ethanol ratio of 1:3 (v/v) for 45 min at 45°C mixing at 150 rpm (Özkan & Bilek, 2015).

2.6. Microwave Assisted Extraction (MAE)

The extraction of phenolic compounds from bay leaf was carried out by the procedure suggested (Zhang et al., 2019) with some modifications. The microwave extraction was carried out using a bay leaf/ethanol (50%), solid/solvent ratio (1:10, w/v), at 500 W power, for 30 s extraction by a microwave oven (Samsung MS23F300EEW/TR).

2.7. Total Phenolic Content (TPC) Analysis

The prepared bay leaf extracts of 0.5 mL were used for total phenols determination. The Folin Ciocalteau reagent was used as an oxidizing agent. The amount of 0.5 mL of extract and 2.5

mL of Folin Ciocalteau reagent (diluted 10 times with water) was mixed for a 4 min, then 2 mL of Na2CO3 (75 g/L) was added to that solution. The samples were incubated at 50ºC for 5 min and then cooled. The absorbance was measured at 760 nm by a spectrophotometer (Cary 50 UV-vis.). The results were expressed in mg GAE/g D.M (Bilek, 2010).

2.8. DPPH Radical Scavenging Capacity

The percentage of antioxidant capacity (AC%) of bay leaf extracts was assessed by DPPH free radical assay (Garcia et al., 2012). The samples were reacted with the DPPH radical in an ethanol solution. The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol, and 0.3 mL of DPPH radical solution (0.5 mM in ethanol). The changes in color were read at 517 nm by a spectrophotometer (Cary 50 UV-vis.). The mixture of ethanol (3.3 mL) and sample (0.5 mL) was used as blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL). The scavenging capacity percentage (AC%) was determined according to Equation 1.

$$AC\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}}\right] \text{ (Equation 1)}$$

2.9. Statistical Analysis

All analyzes were performed in triplicate and the results were given as mean and standard deviation. The difference between the extraction methods based on the total phenolic content and antioxidant capacity was evaluated by the statistical analysis performed with SPSS program (SPSS Inc., Chicago, IL, USA). Significant differences between samples were determined using Duncan's multiple range test at the confidence interval of 95%.

3. RESULTS and DISCUSSION

3.1. The Physicochemical Composition of Dry Bay Leaf

The total dry matter, total ash, and total protein content of dry bay leaf were given in Table 1. In the study of Tainter and Grenis (1993), the total dry matter, total ash, and total protein content of bay leaves were determined as 94.56%, 3.62% and 7.61%. In this context, the physicochemical properties of the bay leaf are compatible with the literature. The bay leaf is a plant rich in ascorbic acid. According to United States Department of Agriculture data, the ascorbic acid content of bay leaves is 46.5 mg/100 g, and it constitutes 77.5% of the daily intake (USDA, 1997). The ascorbic acid content of the bay leaf used in the study was found to be relatively low. This may be caused by the type of bay leaf, the time of harvest and the geographical region where it grows (Molina-Alcaide & Yáñez-Ruiz, 2008).

Table 1. The physicochemical composition of dried bay leaf.

Properties	Dried bay leaf
Total dry matter (%)	95.39±0.39
Total ash (%) (dry sample)	3.67±0.33
Protein content (%) (dry sample)	8.74±0.12
Ascorbic acid (mg/100 g dry sample)	30.43±0.007
Chlorophyll (mg/100 g dry sample)	79.57±0.28

3.2. Comparison of SE, EAE and MAE Methods

The total phenolic content and antioxidant capacity of the extracts were given in Table 2. The enzyme assisted extraction showed the highest efficiency results in terms of total phenolic content and DPPH radical scavenging capacity. It was observed that the Total phenolic content (TPC) extraction yield increased 39.29% comparing with conventional extraction method. By using enzymatic extraction, glycolytic bonds in pectin chain are broken due to the pectolytic activity of Pectinex Ultra SP-L that contains polygalacturonase, pectinesterase and pectin trans-eliminase, hemicellulase, and cellulase, thus the cell wall of bay leaf could be disrupted. The extraction of phenolic compounds can be facilitated with the breakdown of the cell wall (Boulila et al., 2015).

Table 2. Total phenolic content and DPPH radical scavenging capacity of bay leaf by different extraction techniques

Analysis	SE	EAE	MAE
Total phenolic content (mg GAE/g dry sample)	23.29±0.02^{a}	32.45±0.02^{b}	30.49±0.02^{c}
DPPH radical scavenging capacity (%)	36.91%±0.05^{a}	50.72%±0.27^{b}	41.51%±0.09^{c}

a-c- The difference between the groups marked with different letters in the same column is statistically significant ($p < 0.05$).

The microwave assisted extraction (MAE) is also efficient for extraction of phenolic compounds and the extraction yield increased 30.87% according to conventional extraction method. This can be due to in microwave heating, heating was occurred simultaneously, homogeneously, and quickly in contrast to conventional heating. The water inside the cell begins to evaporate with microwave heating. The evaporating water exerts pressure on the cell wall and eventually causes the cell rupture. Thus, the bioactive components pass into solvent and extraction efficiency increases (Mandal et al., 2007). Also, another advantage of MAE shortens extraction time. While the total phenolic content extraction took 24 hours with SE,

MAE was performed in a short time such as 30 s and with higher efficiency. However, the temperature-time relation was important in microwave assisted extraction. The improper relation of temperature-time may cause the degradation of bioactive compounds therefore the efficiency can decrease (Yağcioğlu, 2015). The differences of the extraction methods changed significantly ($p<0.05$). That difference was determined using Duncan's multiple range test at the confidence interval of 95%.

4. CONCLUSION

Overall, the alternative extraction methods comparing to conventional one increased the extraction efficiency. Especially, this study demonstrated that the enzyme assisted extraction was the most efficient method in the TPC extraction and high DPPH radical scavenging capacity. The enzyme assisted extraction revealed 6.43% higher yield than microwave assisted extraction in terms of TPC. However, the fact that enzymes are expensive biological catalysts and the cost of setting up for microwave equipment require feasibility studies of these technologies. Also, there must be a further research about optimization of extraction parameters to improve the extraction efficiency for each method.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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A Review about Honey Effect on Human Body

Balın İnsan Vücuduna Etkileri Üzerine Bir Derleme

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Abstract

Honey is a traditional food which has been used as therapy for many diseases and still essential part of diet. Beside its nutritional properties, honey has many important features such as preventing cancer, neurological and cardiovascular diseases, infections; protecting gastrointestinal system; boosting immune system; alleviating some post-operation complications and correcting hormonal levels. In this study, researches including in vivo and in vitro experiments in addition to human controlled trials examining the effects of honey on each system were included. It was aimed to link these studies' results and traditional practices. Although the researches are sufficient to explain the benefits of honey in general terms, more controlled trial examples are needed on the subject. Promoting accessibility and awareness about medical standardized honeys also aimed in this study. In order to provide these, in addition to informing people, producers should be warned about safe honey production and packaging.

Keywords: Antioxidant effect, Antimicrobial effect, Apitherapy, Honey, Neurological effect, Traditional remedy

Özet

Bal, birçok hastalık için tedavi olarak kullanılan ve halen beslenmenin vazgeçilmez bir parçası olan geleneksel bir besindir. Balın besleyici özelliklerinin yanı sıra kanserden, nörolojik ve kardiyovasküler hastalıklardan, enfeksiyonlardan korunma; gastrointestinal sistemin korunması; bağışıklık sistemini güçlendirmek; bazı ameliyat sonrası komplikasyonları hafifletmek ve hormonal seviyeleri düzeltmek. Bu çalışmada, balın her bir sistem üzerindeki etkilerini inceleyen insan kontrollü denemelere ek olarak in vivo ve in vitro deneyleri içeren araştırmalara yer verilmiştir. Bu çalışmaların sonuçları ile geleneksel uygulamalar arasında

bağlantı kurulması amaçlanmıştır. Yapılan araştırmalar balın faydalarını genel hatlarıyla açıklamaya yetse de konuyla ilgili daha kontrollü deneme örneklerine ihtiyaç duyulmaktadır. Bu çalışmada aynı zamanda tıbbi standardize edilmiş ballar hakkında erişilebilirliği ve farkındalığı teşvik etmek de amaçlandı. Bunların sağlanabilmesi için toplumu bilgilendirmenin yanı sıra güvenli bal üretimi ve paketleme konusunda üreticiler uyarılmalıdır.

Anahtar Kelimeler: Antioksidan etki, Antimikrobiyal etki, Apiterapi, Bal, Nörolojik etki, Geleneksel çare

1. INTRODUCTION

Honey is the only insect product with nutrient value as far as science know. Olaitan et al. (2007) defines honey as natural sweet exudation formed by bees (Apis melifera and Mellifera beecheii) after a complex process consisting of collecting, transforming, combining with spesific enzymes. The process the honey production begins with the collection of nectar, which is a liquiform solution with carbohydrate, amino acid, lipid, mineral composition extracted from nectaries cells of flower (Ball, 2007). This process continues in the bee's gastrointestinal system, nectar encounters many specific enzymes to provide maturation (Graham et al., 1992). Maturation consists of two essential phases; disintegrating sucrose and larger carbohydrates into glucose by enzymes called diastase and invertase and as second phase, removal of excess water by a wing-whisking motion of bees, until water content remain below %20. Another important enzyme, Glucose oxidase contributes to the production of hydrogen peroxide (Oskouei & Najafi, 2013). Nectar is known as determining factor about the flavor and quality of the final product although impossibility of reaching exact data about nectar source. Therefore, a more specific decision can be made about the origin of honeys with distinctive scent and aroma, clover honey or orange blossom honey can be given as example (Graham et al., 1992).

When the chemical properties and ingredients of honey are examined, even though source of nectar causes different compositions, first common finding is acidity of honey. The average pH of honey is measured as 3,9 (Bogdanov, 2009). In a study conducted with 18 different honeys which have different floral source or location the highest pH value was found as 5,13. Though, high sugar content which makes honey a supersaturated solution, prevents acidic taste. The most observed macro minerals can be expressed as K, Ca, Mg, P and Na, respectively (Polat, 2007). Honey contains very small amounts of vitamins, cannot be used as a source (Ball, 2007). Beside maturation enzymes and glucose oxidase, catalase and acid phosphatase are found in enzyme content also (Olaitan et al., 2007). Finally, although polyphenols and proteins are present in very small amounts, the noncovalent and covalent bonds

between them ensures antioxidant and antibacterial properties of honey (Brudzynski, & Maldonado-Alvarez, 2015). In fact, it was determined in the studies that the phenol component provided antioxidant properties to the honey mainly (Khalil et al., 2010). In vivo studies showed that rich-phenol diets have many benefits such as alleviating adverse effects of liver, heart, brain diseases and cancer; other than antioxidant properties (Cianciosi et al., 2018). Apitherapy is an alternative medicine branch, described as the use of honey and honey products for prevention or treatment of various diseases. As written in website of Apitherapy association, apitherapy currently used orally for treatment of; insomnia, anorexia, stomach and intestinal ulcers, constipation, osteoporosis, and laryngitis (American Apitherapy Society, 2019). According to the Gupta and Stangaciu (2014), apitherapy should be handled as a medical concept and be rendered as available to communities especially who are having difficulty in accessing modern health facilities due to hospital costs and lack of insurances. Thus, by popularizing a traditional method, a suitable type of healthcare becomes available without any significant challenges.

The earliest record of honey's use for health purposes is a clay tablet found in the Euphrates Valley, thought to have a prescription for skin infection or ulcer therapy so far (Kramer & Levey, 1954). Frequent use of honey Ancient Egypt's hieroglyphic prescriptions is noticeable, Smith papyrus (between 2600 and 2200 BC) and The Ebers Papyrus (550 BC) can be given as example (Crane, 1999). In these prescriptions, honey is indicated as a topical therapy for infected wounds and many other diseases. Further, honey use for contraception continues as a tradition in Egypt since these prescriptions (Crane, 1999). Ayurveda is a belief system which is grounded on knowledge of life in India, Indus Valley; is another concept that uses honey for remedies widely (1000 BC) (Telles et al., 2007). In Ancient Greek, honey was a popular treatment used for gout and nervous disorders, consumed as a beverage made with unfermented grape and honey (oxymel) (Zumla & Lulat, 1989). In Roma, it is known that pneumonia, pleurisy and snakebites could be treated with honey (Bansal et al., 2005).

Honey existed as precious product with many pharmacological properties in history. Today these traditional remedies continue to be developed and used. Especially in areas which are distant from modern health services with unfavorable economic conditions, traditional healers use honey and hive products frequently. Nepal, African countries, and Eastern Europe can be given as examples (Jones, 2009).

2. PROPERTIES of HONEY on DIFFERENT BODY SYSTEMS

2.1. Anticancer Property of Honey

Cancer is one of the considerable causes of death in the world. According to the National Cancer Institute data (2020), in 2018, 18.1 million people diagnosed with cancer and 9.5 million people died because of cancer and related conditions. Lifestyle habits, smoking and tobacco use, obesity, diabetes mellitus and chronic infections accelerates cancer (National Cancer Institute, 2013). Cancer eventuates after a complex multistep process which includes proliferation, invasion and metastasis. While carcinogens, promoters and inflammatory agents triggering this process; apoptotic proteins, cell cycle proteins, protein kinases and adhesion proteins attempts to control modulation. Despite specific targeted therapies are being searched and has been started to use, common treatments for cancer consists of; surgery, radiotherapy, and chemotherapy although they have all serious side effects (Waheed et al., 2019). Di Bella submitted a new approach to the cancer therapy in 1990s. This approach covers use of honey as complementary natural substance together with anticancer drugs because of immune-modulating and anti-inflammatory effects of honey (Badolato, 2017).

Honeys effect on cancer mechanisms are examined under 6 groups (Waheed et al., 2019). Firstly, cell cycle disruption is primary predisposing factor for cancer. Cell cycle is normally strictly controlled by protein kinases but when they cannot control the cycle rapid proliferation occurs (Pichichero et al., 2010). Cell cycle arresting is a method to stop the process. In recent studies, inhibition of cell cycle at G1/G0 checkpoint by phenolics and flavonoids was observed in melanoma, glioma, colon cancer and non-small cell lung cancer (Afroz et al., 2016). Second important effect of honey is activation of the mitochondrial pathway and outer membrane permeabilization by releasing the cytochrome-C to induct MOMP (mitochondrial outer membrane permeabilization) (Nassar et al., 2016). Apoptosis induction is needed for the death of cancer cells that are increasing in uncontrolled manner. Honey activates apoptosis pathways by providing membrane depolarization and elevating caspase 3 and various proapoptotic protein levels (Ren et al, 2012). As an example, an in-vivo study conducted by application of honey and aloe vera to Wistar rats, decrease of Bcl-2 and increase in Bax protein are observed (Yaacob et al., 2013). Another in-vitro research studied on human breast adenocarcinoma cells (MCF-7) by treating cell lines with acacia honey, showed inhibition in time and dose dependent manner by activation of caspase 9 and increasing caspase 3/7 activity (Portokalakis et al., 2016).

Honey modulates oxidative stress thereby contributes inhibition of proliferation. Since oxidative stress also causes cardiovascular diseases and inflammatory conditions honey can also be used for their treatment (Araujo et al., 2011). Because of N-oligosaccharides (NOS)

component and SCFA (short chain fatty acid) that occurs as a result of digestion, honey has immuno-modulatory effects (Chepulis, 2007). Therefore, swelling caused by malignant transformation can be cured by honey (Dao et al., 2004). Malignancies due to chronic infections such as Hepatitis and EBV viruses can be prevented also. Last benefit of honey which helps combat against cancer is modulation of insulin signaling and estrogenic activity. In recent studies, gelam honey improved insulin resistance caused by high levels of Nf-Kb in HIT-T15 cells (Erejuwa et al., 2012). Contradictory action of honey helps modulating estrogen receptors and thus honey becomes functional in treatments of estrogen dependent cancers of breasts and endometrium (Erejuwa et al., 2014). Honey also has antimutagenic activity. In a study conducted with seven different honey (acacia, buckwheat, fireweed, soybean, tupelo and Christmas berry), important level of inhibition of Trp-p-1 mutagenicity has been observed (Wang, 2002).

Although exact mechanisms are not known and needed to be searched, most of the benefits of honey are due to the flavonoids and polyphenols it contains and the type and amount of them varies according to the type of honey (Ahmed & Othman, 2013). For example, Coriander honey is effective on Ehrlich Ascites Carcinoma due to mechanisms which decreasing lipid peroxidation and superoxide dismutase, decreasing ascetic fluid volume and increasing Ig M, G and A levels (Hegazi et al., 2015). Tualang honey in Malaysia improved Oral Squamous cell carcinoma, Osteosarcoma, Leukemia and Breast cancer by depolarization of mitochondrial membrane and inducing apoptosis (Ahmed et al., 2017; Fauzi et al., 2011)

Beside its sole use, honey has significant benefits as companion to modern cancer therapies. Several studies show stimulation of chemotherapeutic agents such as cyclophosphamide and 5-fluorouracil (Moniruzzaman, 2012). Honey serves as treatment against adverse effects of chemotherapies which are infections such as oral mucositis and cisplatin induced acute kidney disease also (Porcza et al., 2016; Singh, 2017).

2.2. Antimicrobial Property of Honey

Antimicrobial features of honey have been known since ancient times. Dioscorides stated that honey can be efficient for ulcer therapy in 50 AD and Aristotle (384-322 BC) indicated that pale honey is good for sore eyes and wounds (Molan,1999). Today antibiotics are preferred for treatment of bacterial infections however widely use of them has been causing resistance. As consequence, regimen changes for antibiotics and search for new compounds to replace them are needed (Feás et al., 2013). Since Dutch scientist Van Ketel proved honey ‘s antibacterial activity in 1892, honey has been considered as a treatment for bacterial infections (Eteraf-

oskouei & Najafi,2013). Eteraf-oskouei and Najafi (2013) claimed that because of honey may not develop resistant bacteria even when it used in high doses, it could be consumed continuously. Honeys inhibitory action on Gram-positive and gram-negative bacteria including MRSA (Multi drug resistant strains) and some fungi and viruses are observed in recent studies (Irish et al., 2006; Kwakman et al., 2008; Naama, 2009).

Antimicrobial activity of honey is associated with four different mechanisms which separates honey from antibiotics. First one is high sugar content that contributes dehydration of bacteria by osmosis (Olaitan, 2007). Second one is low pH (3.2- 4.5) (Allen et al., 1991). Third and crucial one is Hydrogen peroxidase produced by glucose oxidase. Glucose oxidase is an enzyme which serves to preservation. (Glucose + H2O + O2 → Gluconic acid + H2O2) (Somal et al., 1994). It worth to mention here, although antimicrobial use of Hydrogen peroxide is common, it causes cellular and protein damage by increasing reactive oxygen radicals. (Cochrane,1991; Simon et al,1981). Last mechanism is effect of flavonoid and polyphenol compounds such as pinocembrin, pinobanksin and chrysin (Wahdan, 1998). Source of antimicrobial effect might be the origin of honey also (Bogdanov et al., 2008).

Honey also contributes defense mechanisms against microbes by boosting immune system. Study of Tonks et al. (2003) shows increased count of T-lymphocytes and B-lymphocytes and active neutrophils in cell cultures as immunologic effect of honey. Tonks (2007) also discovered that Manuka honey increases TNF alpha levels by modulating Toll like receptors. Honey can also stimulate monocytes via releasing cytokines, tumor necrosis factor (TNF)-alpha, interleukin (IL)-1 and IL-6 (Tonks et al., 2001). Further, honey provides maintenance of glycolysis, major energy mechanism of macrophages. With this mechanism macrophages can continue to work in the damaged, oxygen free tissues. Acidity also contributes to the macrophage function by creating acidic environment inside phagocytic vacuole (Molan, 2001).

Modern studies about antimicrobial activity of honey focuses on observing differences between specific organisms. Huttunen et al.'s (2013) study compares effects of 10 different honey samples on bacterial resistant strains such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella enterica serovar Typhimurium*, *Bacillus cereus*, *Bacillus subtilis*, and *Listeria monocytogenes*. Pure honey was more effective, and sensitivity of different bacterial strains were different. In general, all honey samples demonstrated antibacterial activity and inhibition of bacterial growt. According to study by Wasihun and Kasa (2016), honey has potential bacteriostatic and bactericidal activities

on multidrug resistant human pathogenic bacterial isolates (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, coagulase-negative *Staphylococcus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*). Alandejani et al. (2009) showed that bactericidal rates of Sidr and Manuka honeys were higher than different antibiotics (cefazolin, oxacillin, vancomycin, azithromycin, fusidic acid, gentamicin, and linezolid) against methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), or *Pseudomonas aeruginosa* (PA) biofilms.

Standardization and safe medical use of honey is difficult because of different sensitivity of bacterial strains and changes in antibacterial property for different honey samples (Grego et al., 2016; Sousa et al., 2016). However, by controlled production and analyzing batches to measure bactericidal activity, Revamil source honey and Manuka honey have been used for medical occasions (Knight, 2013).

Ceyhan and Ugur (2001) compared antimicrobial activity of 84 honey samples with different floral sources on 8 bacterial and 2 fungal strains. Honey sourced from thyme, pine and carob were more effective. As a result, most of honey samples showed inhibitory effect on bacterial growth. Fungi were less sensitive than bacteria. Honeys antimicrobial effect on fungi reported on some yeast and species of *Aspergillus* and *Penicillium*, dermatophytes, *Candida albicans*, Cutaneous and superficial mycoses like ringworm and athlete's foot (Kumar et al., 2010). Inhibition of fungal growth and toxin production by pure honey is observed in the study of Al-Waili and Haq (2004). Inhibition of bacterial growth also contributes to inhibition of secondary fungal infections. Some studies revealed topical application of honey can be used as treatment for seborrheic dermatitis and dandruff also (Zaidi et al., 2019).

Antiviral property of honey investigated on herpes lesions, as result it has been seen that topical application is more beneficial for treating recurrent attacks of labial and genital herpes lesions compared acyclovir cream (Al-Waili & Haq, 2004). Honey also has inhibitory effects on *Rubella virus* activity. (Eteraf-Oskouei, & Najafi, 2013). Some studies which show Manuka Honey's dose dependent inhibitory activity on HIV-RT exist also (Shikamoto, 2021).

In a study, it was determined that 10% honey concentration applied to the Ecinococcus granulosus parasite, which causes hydatid cyst (echinococcosis), has a lethal effect from the third minute (Karadal & Yildirim, 2012). Antileishmanial property of honey is observed in some studies (Bassam, 1997). In a study conducted to determine the antimicrobial effects of honey samples collected from Bingöl region, it was stated that 0.1 mL honey sample inhibits the growth of bacterial species such as Escherichia coli, Staphylococcus aureus, *Klebsiella*

pneumoniae, *Bacillus brevis*, *Pseudomonas aeroginosa* and fungi species such as *Candida albicans* and *Rhodotorula rubra* (Aksoy & Digrak, 2006).

Mohammed Ali and Kunugi (2021) stated that honey and honey added herbal remedies can have positive effect on COVID-19 due to their flavonoid components, especially naringin. Acceleration of healing is thought to be depended on reduced oxidative stress and immune boost.

2.3. Gastrointestinal Effects of Honey

Gastrointestinal diseases such as gastroenteritis, diarrhea, gastritis and peptic ulcer are common throughout the world. As claimed by studies, honey can be therapeutic for these diseases because of its antimicrobial and gastroprotective functions. The most common cause of diarrhea is gastroenteritis, infection of the gastrointestinal tract. *Salmonella, Shigella and Enteropathogenic E. coli* constitutes most frequent gastroenteritis agents (Pawlowski et al., 2009). Study of Jeddar et al. (1985) shows that honey has bactericidal effect on many pathogens including *Salmonella, Shigella and Enteropathogenic E. coli*. A clinical study on infantile gastroenteritis demonstrates honey lessens time of bacterial diarrhea caused by *Salmonella, Shigella and Enteropathogenic E. coli* in patients. Although honey was successful in shortening time of bacterial diarrhea, no change was observed in viral gastroenteritis (Haffejee & Moosa,1985). During these infections, first step is defined as attachment of bacteria to mucosal epithelial cells, therefore inhibition of attachment is important for prevention (Alnaqdy et al., 2005). Honey contributes this inhibition by several mechanisms; by coating bacteria, bacterial electrostatic charge or hydrophobicity change and antibacterial properties (Alnaqdy et al., 2005; Edebo et al., 1980; Sakai et al., 1987).

According to WHO, as routine therapy for diarrhea, electrolyte solution with glucose is recommended (WHO, 1976). In a clinical study, effect of honey replacement instead of sugar in solution has been observed. It was found that honey was effective (Haffejee & Moosa,1985). Honey also help battle against gastroenteritis by repairing damaged intestinal mucosa, stimulating growth of new tissues and with its anti-inflammatory action it corrects malfunctioning in mucosa and prevents serum loss (Molan, 1999).

According to the study of Ali et al. (1991), %20 honey solution inhibits *Helicobacter pylori* isolates which are the main cause of peptic ulcer disease, including isolates which are resistant to antibiotics. In studies conducted on gastrointestinal infections (gastritis, duodenitis, gastric ulceration) caused by bacteria and rotavirus, it has been observed that oral treatment of

honey is effective (Somal et al., 1994; Topham., 2002). Antibiotic property is not the only mechanism of honey that assists preventing gastritis and peptic ulcer disease. Reduced secretion of gastric acid and increased blood supply by stimulating sensory nerves and releasing vasodilatory peptides are another two significant protective effect of honey (Ali & Al-Swayeh, 1996; Al-Swayeh & Ali, 1998).

A study indicated %80 recovery rate of 600 gastric ulcer patients who treated with honey (Kandil, 1987). Study of Ali et al. (1991) demonstrated dose dependent action of honey on peptic ulcers caused by alcohol and indomethacin (aspirin type anti-inflammatory drug). Nasutia et al. (2006) recommended using honey like sucralfate because of its healing effect on lesions induced by indomethacin and ethanol.

Prebiotics described as non-digestible dietary supplement which balances intestinal microbiota by increasing beneficial organisms. Both in vivo and in vitro studies showed positive effect of honey on growth of beneficial bacteria (*Bifido bacteria and lactobacilli*) due to its oligosaccharide components (Sanz et al., 2005). Honey is accepted as convenient sweetener for fermented milk products which contains beneficial bacteria such as *Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus delbruekii* and *Bifidobacterium bifidum* (Bansal et al., 2005). Study of Coskun and Dirican (2019) showed that pine honey considerably increased prebiotic property of yoghurt beside providing better structural characteristics. Numbers of *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. acidophilus* are observed beyond 10^6 cfu/g which is urged minimum level.

2.4. Cardiovascular Effects of Honey

Cardiovascular diseases constitute significant part of illnesses as deaths in the developed countries. Arrythmias, myocardial infarctions seen as symptoms of ischemic heart disease. One of the risk factors for cardiovascular diseases have been indicated as oxidative stress (Kasper, 2015). Several in vivo and clinical studies showed increased levels of vascular superoxide anion production in the states of hypertension, hypercholesterolemia and hyperlipidemia. (Guzik et al., 2000; Miller et al., 1998; Morawietz et al., 2001). Oxidized LDL-C can easily be invaded and internalized by macrophages to cause atherosclerotic plaques by forming foam cells (Palinski et al., 1989). Currently, for the treatment of such diseases drug therapy is preferred. Contrarily, because of the cardiovascular disease's complexity and interconnected structure, patients experience limitations and adverse effects while using especially anti-arrhythmic drugs (Hume & Grant, 2007).

Honey has been used as a treatment of several diseases since ancient times, but cardiovascular use is not included in traditional prescriptions (Ahmed et al., 2003). Currently, many in vivo studies focus on cardiovascular effects of honey. As result of these studies, cardioprotective effects of honey are better known; Inhibition of atrial fibrillation, anti-arrhythmic, anti-thrombotic, antiatherogenic, relaxation of blood vessels, reduction in blood pressure, inhibition of LDL oxidation, improved dyslipidemia. All these mechanisms mainly ground on antioxidant effect of honey (Bahrami et al., 2009; Schramm et al., 2003; Yaghoobi, 2008).

Honey increases antioxidant agents such as vitamin C, Beta-carotene, uric acid and glutathione reductase in body (Khalil & Suleiman, 2010). Antioxidant capacity depends on botanical source of honey, according to a study antioxidant level is higher in darker honeys (Baltrusaityte et al., 2007). NO metabolites of honey can also contribute to the vascular protection (Bogdanov et al., 2008).

Yaghoobi et al. (2008) measured cardiovascular risk factors such as body weight, body mass index, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triacylglycerol, fasting blood glucose (FBG) triacylglycerol and C-reactive protein (CRP) on 55 patients who were overweight or obese. As result, mild reduction in body weight and body fat were observed. While honey was reducing total cholesterol, LDL-C, triacylglycerol, FBG, CRP and increased HDL-C in patients with normal values; total cholesterol, CRP and LDL-C reduced more in patients who have elevated values.

Najafi et al. (2008) recommended honey as prophylaxis as a result of their study where they searched effect of honey on ischemia/reperfusion (I/R) induced injuries in isolated rat heart by perfusing enriched Krebs solution with natural honey for 10 min before to 10 min after ischemia. Another in vivo study conducted on anesthetized, normal or stressed rats demonstrated natural honey pretreatment provides protection against epinephrine-induced vasomotor dysfunction and cardiac disorders and preserved the positive inotropic effect of adrenaline (via enzymatic and non-enzymatic antioxidants, minerals and NO release from endothelium by vit C influence) (Rakha et al., 2008). In a prospective study that has observed CHD and stroke mortality in postmenopausal women in Iowa, beneficial effect of flavonoid intake was observed (Yochum et al., 1999). A phenolic compound (Salvia miltiorrhiza extract), was founded helpful in prevention of postoperative increase of endothelin-1 which increased after cardiopulmonary bypass in children with congenital heart defects (Xia et al., 2003).

2.5. Respiratory Effects of Honey

Antibiotics are prescribed for upper respiratory tract infections primarily (Goossens et al., 2005). Although most URTI have viral source and because of the alternative treatments are inadequate, health professionals continue to prescribe antibiotics (Hersh et al., 2013; Kenealy & Arroll, 2013). Since honey is known as therapy for respiratory infections in history, many modern studies have been performed to confirm this popular belief and present honey as replacement for antibiotic therapy. According to a meta-analysis by Abuelgasim et al. (2021) honey improves URTI symptoms compared to usual care and reduces combined symptom score (which includes symptoms like sore throat), cough frequency and cough severity. Cough is more important concern in children because of their immature immune system. (Kantar, 2016). Paul et al. (2007) stated honey is helpful for symptomatic remedy of nocturnal cough and sleep difficulty associated with childhood upper respiratory infections. They recommended honey as cough treatment also. Another study about honey's effect on acute symptoms of URTI conducted on Malaysian Hajj Pilgrims. As result, significant decrease in sore throat rates observed in the people who consume Madu Lebah Tualang - Agromas honeys for 3 weeks,20 g, twice, daily (Suleiman et al., 2011). Study of Kilty et al. (2010) focuses on treating Chronic rhinosinusitis (CRS) which is a disease caused by biofilm producing bacteria. Chronic rhinosinusitis (CRS) is normally treated with antibiotics but in patients who has undergone endoscopic sinus surgery topical therapy is preferred. In this study manuka honey has been found efficient against bacteria without causing any histological injury to the olfactory epithelium. Kamaruzaman et al. (2014) have observed aerosolized honey's effects on ovalbumin induced chronic asthma in rabbit model. Significant inhibition in goblet cell hyperplasia and mucus overproduction were seen. Anti-inflammatory property of honeys such as Gelam, Buckwheat and Manuka is thought to be the reason for relief of asthma symptoms. Manuka honey is notified as booster for TNF-α, IL10, IL-1ra, PDG and TGF-β.26.

2.6. Metabolic Effects of Honey

Honey use instead of refined sugars is safe for diabetic patients, it has been used as remedy for diabetes since early ages (Katsilambros et al., 1988). There can be two primary reasons; firstly, smaller amounts of honey provide more sweet taste and secondly, honey has less calories with much more nutrients such as minerals and vitamins. In studies, increased insulin secretion stimulated by honey, decreased blood glucose and increased hemoglobin with advanced lipid profile were observed (Al-Waili, 2003; 2004). Honey also reduces hepatic transaminases, triglycerides and glycosylated hemoglobin (HbA1c) and increases HDL cholesterol

(Busserolles et al., 2002; Erejuwa et al., 2009). Another benefit of honey about diabetic use is its antioxidant property, it has been proved that β-cell dysfunction influenced insulin resistance is caused by oxidative stress (Drews et al., 2010). In previous studies, some controversial results have been observed. Although studies conducted with Dutch Gold honey showed no improvement in glycemia and insulin sensitivity caused by cytokines (IL6), in vivo studies which were using Tualang and Nigerian honey demonstrated improvement in glycemia and dyslipidemia (Erejuwa et al., 2009; Raatz et al., 2015). This might be caused by polyphenols in honeys with tropical source or several different enzymes such as α-glucosidase inhibitors and dipeptidyl peptidase-4 inhibitors stimulated by bee (Erejuwa et al., 2012).

2.7. Wound Healing Effect of Honey

Wound healing is well known effect of honey since it has been started to be used for medical purposes. Prevention of wound infections by honey was effectively used by Russians in World War I and Germans discovered a remedy with mixing cod liver, oil and honey for ulcers, burns, fistulas and boils (Bansal et al., 2005). Honey has been searched on several type of ulcers, wounds and skin diseases and found to be helpful for all. Although honey is effective on all kind wounds, degree of effect varies between types and severities. Applying and removing honey dressings don't create a challenge like other materials. Wounds must be covered with adequate amount of honey and cavities should be full filled. (Bansal et al., 2005; Molan, 1998; Molan & Brett,1998). For removing, simple bathing is enough (Molan & Rhodes, 2015). Reasons of honey's wound healing property can be explained by its antimicrobial and anti-inflammatory effects. Honey also regenerates tissues with direct nutrient effect and by creating osmotic outflow it helps eliminating dirt and debris from wound. Hydrogen peroxide also helps fibroblast proliferation and angiogenesis by VEGF release under influence from macrophages (Oryan et al., 2016). Another contribution of honey is its ability to eradicating bacteria while creating moist environment and eliminating bad smells. By adding honey dressing to usual therapy, surgical costs and hospital stay can be diminished (Efem, 1988).

Majtan et al. (2010) observed elevated production of mediators such as cytokines (TNF-alpha, IL-1beta and TGF-beta) and matrix metalloproteinase-9 (MMP-9) from keratinocytes; MMP-9 influenced degradation of type IV collagen in the basement membrane after honey incubation. Shamloo et al. (2021) produced a Chitosan/Gelatin/PVA hydrogel enriched with honey and observed product's effect in in vitro and in vivo studies. Honey containing gels increased cell growth and contributed to preservation of well-structured layer of epidermis containing mature collagen. Honey founded effective especially on second degree

partial burns (Pramesty, 2021). Jain (2021) informed that they are using medical grade Indian honey for patients who has diabetic foot in their Diabetic Foot and Wound Care clinic. Another study on ulcers has been searched on oral ulcers in the free gingival graft (FGG) donor site. Because of post-operative pain and high costs of periodontal dressings and acrylic stents, honey was promising as an alternative. Ziziphus lotus honey substantially increased number of blood vessels and epithelization rate on 14th day (Golpasandhagh et al., 2021).

Honey also used for solving oncology patients' problems such as infection free post-operative wounds after radical surgery of vulva carcinoma and treating radiation induced mucositis (Cavanagh et al., 1970; Motallebnejad et al., 2008). In a study conducted on Fournier's gangrene, it has been showed that, honey accelerated healing with decreased edema and scarring. Decrease in mortality was observed also (Haidari et al., 2014). Honey has been applied as topical remedy to patients with postoperative wound infections following caesarean section or hysterectomies, bacteria are eliminated, and wound healing is improved without any scar formation (Al-Waili, 2005).

2.8. Oral Health and Honey

Honey have been used for maintenance of oral health because of its multifunctional form (Ramsay et al., 2019) Firstly, plaque forming can be inhibited by honey because of its antimicrobial property. In previous studies, *S. mutans*, *P. gingivalis* and *L. acidophilus* counts reduced with chewing honey (Atwa et al., 2014; English et al., 2004). Thus, honey was presented as periodontal protection (Chapple., 1996; Samani et al., 2011). Hidaka et al. (2008) observed anti-calculus effect (inhibition of oral calcium phosphate) of honey. Significant advancements were observed with topical application of honey to Lichen planus and lesions of desquamative gingivitis (El-Haddad & Al-Shawaf, 2013). Honey application on dental abscesses and chronic osteomyelitis also showed improvement in a study conducted on 10 dental infection cases (Elbagoury & Rasmy, 1993). Bulut and Tufekci (2016) observed honey activity on chemotherapy induced oral mucositis in pediatric patients. As result significant decrease observed in patients who honey applied before mucositis occurred compared to group which honey applied after mucositis occurred.

2.9. Ophthalmological Effects of Honey

Ophthalmological use of honey was recorded in Attica, Greece and India first (Molan, 1999). In India, honey containing eye drops still being used for several eye diseases (Mahawar& Jaroli, 2006). Further in an Indian Medical College, corneal ulcers precipitated by bacteria were treated by topical application of honey (Ajibola et al., 2012). Malian locals use natural honey to prevent

eye scarring caused by measles infection (Imperato& Traoré, 1969). Topical natural honey application accelerated epithelial healing on injured corneas (KerWoon et al., 2015). Another study conducted in vivo showed equal activity compared to usual treatments, by both oral and topical Tualang honey use on alkali chemical injury on rabbit's cornea (Bashkaran et al., 2011). Jankauskiene et al. (2007) observed that polyfloral honey eye drop treated dry eye syndrome when it is applied early stages of disease. Cernak et al. (2012) examined honey as antimicrobial prophylaxis for cataract surgery and vitrectomy patients. No difference observed between topical applications of 25% honey solution and 0.3% ofloxacin. Synergistic activity between manuka honey and oxacillin, tetracycline, imipenem, and mupirocin against methicillin-resistant *Staphylococcus aureus* (MRSA) has been found (Jenkins & Cooper, 2012). Bullous keratopathy is a disease with swollen cornea which caused by cataract surgeries usually (Goncalves et al., 2008). Hypertonic saline is used as treatment because of its osmotic effect, honey is suggested as replacement (Knezovic, 2006). In a study, healing effect of honey was observed by applying 4-5 drops daily, epithelial edema was eliminated, corneal bulla dissolved in both eyes (Mansour, 2002). Flavonoid component of honey has been found effective for cataract due to several pathways such as decreasing eye lens opacification via inhibition of oxidative stress, epithelial cell signaling, lens calpain proteases (Stefek & Karasu, 2011). Flavonoids also found helpful for glaucoma (Stefan et al., 2011).

2.10. Neurological Effects of Honey

Although honey has been searched as a therapy for many diseases such as gastrointestinal disorders cancer wounds, knowledge about relationship of neurology and honey remained limited. However, we can conclude neurological effects of honey according to results of in vivo experiments. Opinions on honeys beneficial effects on nervous system derive from ancient texts for example Ayurvedic formulation which given to boosting memory and concentration, maintaining strength for longer life (Mishra, 2011). Modern medicine focuses on neurologic development, memory, degenerative diseases and behavioral disorders while searching effects of honey.

First found trait is honey contributed to postnatal development of nervous system in newborn babies and preschool age children by neurogenesis mechanism in hippocampus and cerebral cortex. With this contribution, advanced memory and growth, decreased level of anxiety and increased intellectual performance is observed after a while (Oyefuga et al., 2012). However, adding honey to the diet during first year of life is not recommended because of

immune deficiency against botulinum toxin (Tollofsrud et al., 1998). Another experiment on postmenopausal women showed progress in immediate memory (Othman et al., 2011).

Antioxidative property of honey is one of the earliest discoveries about its benefits. Antioxidative process in nervous system includes decreased lipid peroxidation as result of superoxide dismutase (SOD) and glutathione reductase activity and thus free radical level is decreased (Oyefuga et al., 2012).

Oxidative stress causes inflammatory response, apoptotic response and necrotic response that eventuates as neuronal cell death and aging. Cai et al. (2011) showed honeys antidegenerative effect on hippocampal C1 region which is sensitive to oxidative stress. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis are the subjects which researchers focus on currently (Mariani et al., 2005).

In vivo studies on rats helped discovering many behavioral and cognitive benefits of honey. A study showed decreased anxiety and increased spatial memory by measuring object recognition in honey fed rats compared control groups (Chepulis et al., 2009). Another study demonstrated increased level in exploratory, rearing and grooming activities in dose dependent manner (Oyekunle et al., 2010). Akanmu et al. (2011) experimentalized honey fed rats in tests such as Y-maze test, pentobarbital induced hypnosis, hole-board and elevated plus maze tests, picrotoxin seizure model, tail flick test and forced swimming test. As a result of this study memory boosting, anxiolytic, antinociceptive, anticonvulsant, and antidepressant effects of honey are observed, respectively. Further, honeys effect on glial cells beside of neurons were observed in a rat model with cerebral focal-induced ischemia (Galindo et al., 2011). This information added a new dimension to the neurodegenerative studies and neuronal injury associated with stroke.

Flavonoids and polyphenols which has neuroprotective effects can be stated as; Apigenin, Caffeic acid, Catechin, Chlorogenic acid, Chrysin (5,7-dihydroxyflavone, *p*-Coumaric acid, Ellagic acid, Gallic Acid, Luteolin and Naringenin (Rahman et al., 2014).

3. COMPARISON of PROMINENT FEATURES between DIFFERENT HONEYS

Honeys can have different properties because of their source, species of bee which producing that particular honey, season or place (Hoffman et al., 2021). Alvarez-Suarez et al. (2018) explained that, when honeys produced by *M. beecheii* and *A. mellifera* were compared; more activity against bacterial and especially yeasts such as Candida salbicans due to higher value of

bioactive compounds was observed in honeys produced by *M. beecheii*. Manuka honey has an important place in literature because of its well-known antimicrobial and antioxidant properties. However, previous studies proved that there might be other honeys with better abilities. In a study, it is indicated that, *M. beecheii* honey demonstrates more potent inhibitory activity against both Gram-positive, Gram-negative bacteria samples and *C. albicans* than other honeys including Manuka, with its non-specific action (Morroni et al., 2018). Another study conducted with several honeys such as Malaysian longan and rubber tree honeys, gelam and manuka honey; showed that sourwood honey has highest antioxidant effects (Moniruzzaman et al., 2013). It has been indicated that different honeys activate diverse pathways; for example, while Manuka and buckwheat activate p38, ERK1/2, and mTOR pathways than acacia, the PI3K pathway is mostly stimulated by manuka (Ranzato et al., 2012). A study stated since antimicrobial activity of Agastache honey (Figure 1) is close to Leptospermum (Figure 2) honey (Manuka), it can be used in topical applications for preventing wound infections (Anand et al., 2019). *Melipona marginate* honey is also used in studies and its effects are observed as reducing ear edema and decreasing myeloperoxidase activity (Borsato et al., 2014). Study of Malkoc et al. (2019) revealed that Blackthorn (Paliurus spina-christi Mill.) (Figure 3) honey which is a monofloral honey limited to Marmara Region of Turkey has high antimicrobial and antioxidant properties.

Figure 1. Agastache

Figure 2. Leptospermum

Figure 3. Paliurus spina Christi

4. DETRIMENTAL EFFECTS of HONEY

Honey is a food that should be considered while it is consumed because of several adverse effects. Although allergy is unusual, it can be caused from both pollen and bee proteins (Bansal et al., 2005). A case report notified an anaphylactic reaction to honey (Aguiar et al., 2017). Honey might be affected easily by environmental pollutants such as pesticides, heavy metals, antibiotics and substances used in beekeeping (Bogdanov, 2006). Another risk is botulism due to *Clostridia* spores which can be killed by gamma irradiation (Molan & Allen, 1996). Risk is more for infants below 1 year of age (Eijlander, 2011). In topical application, stinging pain or

burning sensation in wounded diabetes patients due to dehydrated tissues caused by high blood glucose can be observed (Simon et al., 2009). Lethality of Mad honey is well known. Reason for this lethality is Grayanotoxins, particularly Andromedotoxin founded in Ericaceae (Figure 4) plants (Hikino et al., 1979; Yavuz et al., 1991). Grayanotoxin activity is based on mechanism of prevention of inactivation by binding Sodium channels in body. Bradycardia, hypotension, nausea, vomiting, sweating, salivation, dizziness, weakness, loss of consciousness, fainting, blurred vision, chills, general convulsion and cyanosis are seen (Ozhan et al., 2004). Onat et al. (1991) revealed that bradycardia due to mad honey poisoning can be treated by atropine sulphate which is muscarinic receptor antagonist. Though one spoon of mad honey is enough to cause toxication, it still has been used as treatment of gastrointestinal system disorders, hypertension and as sexual stimulant by local people (Mutlu et al., 2017). For preventing deaths caused by mad honey and others, bioactive components must be specified and indicated by producers and sellers. Health professionals must take patient's history detailed and carefully (Ozhan et al., 2004). Fake honey is a method to reduce expenses and easing the processes of beekeeping. Production is carried out in various ways, such as feeding bees with sugar syrups instead of nectar or secretion, producing honey from these syrups or adding sugar syrup directly to honey. Honey forgery can be detected by various techniques such as determining the honey's proline content, potassium and sodium ratio (K / Na), and total pollen spectrum. If honey's source is specifically important for treatment, this situation should also be paid attention (Mutlu et al., 2017).

Figure 4. Ericaceae

5. CONCLUSION

Honey is a very nutritional food with many beneficial properties to health. Although these properties are confined to mainly antimicrobial, antioxidant, immune booster and nutritional regulating effects of honey for now; it is obvious that honey is far more beneficial than science know. Honey must be appreciated and its other specific activities on systems should be detailly defined in turn its use in medical areas can be expanded. Since ancient times, honey is seen as miracle; health experts can build a bridge between traditional and modern medicine by utilizing

honey. It shouldn't be forgotten that each honey has its own properties like bioactive components, activity in body, microorganisms, and toxins. Therefore, for both table consuming or with medical purposes, each party must be analyzed and labeled. Processes of production of honeys which are thought to be significantly beneficial for medical use and must be disseminated, must be standardized in terms of determining dose influenced by its bioactive component levels.

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DECLARATIONS

The authors declare that they have no conflicts of interest.

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The Antiviral Effect of the Propolis from Turkey on the Replication of Autographa californica nuclear polyhedrosis Virus (AcNPV) Developed in Spodoptera frugiperda Cell Culture

Spodoptera frugiperda Hücre Kültüründe Geliştirilen Autographa californica nükleer polihedrosis Virüsünün (AcNPV) Replikasyonu Üzerine Türkiye Propolisinin Antiviral Etkisi

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Abstract

In this study, the influence of propolis extracts gathered from Turkey on the replication of *Autographa californica nuclear polyhedrosis* virus (AcNPV) developed in *Spodoptera frugiperda* culture was researched through examining changes in cytopathic influences, hydroxyl radical mediated DNA damage and progeny virus concentration. By making use of propolis extract, we found inhibiting effect on cytopathic changes and DNA damage. It was found that propolis extracts on the cytopathic changes and hydroxyl radical-mediated DNA damage were similar to the control. The results showed that propolis has a protective effect on DNA exposed with H_2O_2. In terms of the production of occlusion bodies in Sf9 cells, no important difference was found between the average percentage of contaminated cells in contaminated control cells when the concentration of propolis is 3.125, and 1.5625 µg/mL (4.018 (Plaque forming units (PFU) pfu/mL and 5.145 pfu/mL respectively) ($p<0.05$) and no significant difference was found between the average number of occlusion bodies in the contaminated control cells and the contaminated cells in the concentration of propolis. Respectively 98.70% and 98.67% reduction were found in virus titter with propolis extracts when the concentration of propolis extracts 100, 50 µg/mL were included 1 h p.i. ($p<0.05$). However, different concentrations of propolis extract reduced the number of progeny viruses significantly.

Keywords: *Baculovirus*, Propolis, Antiviral activity, Virus replication

Özet

Bu çalışmada, *Spodoptera frugiperda* kültüründe geliştirilen *Autographa californica* nükleer polihedrozis virüsünün (AcNPV) replikasyonu üzerine Türkiye'den elde edilen propolis ekstraktlarının etkisi, sitopatik etkiler, hidroksil radikal aracılı DNA hasarı ve progeni virüs konsantrasyonundaki değişiklikler incelenerek araştırılmıştır. Propolis özü kullanarak sitopatik değişiklikler ve DNA hasarı üzerinde engelleyici etki bulduk. Propolis ekstraktlarının sitopatik değişiklikler ve hidroksil radikal aracılı DNA hasarı üzerinde kontrole benzer olduğu bulundu. Sf9 hücrelerinde emilim bölgesi üretimi açısından, propolis konsantrasyonu 3.125 ve 1.5625 µg/mL olduğunda kontamine kontrol hücrelerinde kontamine hücrelerin ortalama yüzdesi arasında önemli bir fark bulunmadı (sırasıyla 4.018 (plak oluşturan birim) (PFU) pfu/mL ve 5.145 pfu/mL) ($p<0.05$) ve kontamine kontrol hücrelerindeki ortalama emilim bölgesi sayısı ile propolis konsantrasyonundaki kontamine hücreler arasında anlamlı bir fark bulunmadı. 100, 50 µg/mL propolis ekstraktlarının konsantrasyonu 1 h pi dahil edildiğinde propolis ekstraktları ile virüs titresinde sırasıyla %98.70 ve %98.67 azalma bulundu. ($p<0.05$). Bununla birlikte, farklı propolis özütü konsantrasyonları, virüslerin sayısını önemli ölçüde azaltmıştır.

Anahtar Kelimeler: *Baculovirus*, Propolis, Antiviral aktivite, Virüs replikasyonu

1. INTRODUCTION

It is acknowledged that baculoviruses are a body of enveloped double-stranded DNA viruses affecting arthropods, especially insects belonging to the order Lepidoptera (Bilisard et al., 2000). The *Autographa californica* nuclear polyhedrosis virus (AcNPV) is an insect virus belonging to Baculoviridae family. In some Baculoviruses, virus enhancing factors (VEFs) in OBs improve infectivity (Bischoff & Slavicek, 1996; Wang et al.,1994). Following ODV envelope's direct blending to midgut columnar epithelial cells' plasma membrane (Granados and Lawler, 1981; Horton & Buand, 1993), the virions become uncoated and transferred to the nucleus and progeny nucleocapsids are formed in the nucleus. Later, progeny nucleocapsids move to the plasma membrane from the nucleus and grow into the hemocoel by means of basal sides of epithelial cells. In the hemocoel, before causing the death of the host, BVs first affect the tracheal epithelium and hemocytes and later affect the fat body, muscle, Malpighian tubules, and other tissues (Engehard et al., 1994). Due to its pharmaceutical characteristics, propolis (bee glue), a resinous beehive product, has been used since classical times (Matsuda, 1994). Bees use propolis to secure the walls and entrance of the hive and dead invaders. The best known and the most researched biological activities of propolis are antiviral, antifungal, anti-inflammatory, antibacterial, wound healing and antitumor activities (Marcucci, 1995). It is a natural remedy that has been in use for centuries (Castaldo & Capasso, 2002) and is widely applied in traditional medicine thanks to its pharmacological benefits of anticancer, antioxidant

(Kumazawa et al., 2004), antiviral, anti-inflammatory and antimicrobial properties (Banskota et al., 2002). Propolis is useful in foods. Propolis is as a natural preservative and a source of bioactive compounds for foods and drinks that help improve shelf-life and consumer health (Duman & Ozpo, 2015). The present study researched the influence of propolis extracts gathered from Turkey on the replication of *Autographa californica* nuclear polyhedrosis virus (AcNPV) in *Spodoptera frugiperda* cell culture through examining changes in cytopathic influences, hydroxyl radical mediated DNA damage and virus concentration (Bischoff & Slavicek, 1996; Wang et al.,1994).

2. MATERIALS and METHODS

2.1. Propolis Extract Preparation

Propolis extracts prepared such as represented by Boeru and Derevici (1978). Propolis sample was collected from colonies of honeybees located in the north-east Black Sea Region in Turkey. Hand gathered propolis was maintained in a dry place and reserved at -20°C until its used process. 200 grams of propolis were frozen to -20 °C, slaughter in small pieces, and ground in a frozen mortar then 20% ethanol extract propolis was prepared by supplementation 200 gram of propolis to 800 mL of 70% ethanol (1: 4 w/v) in a shaker at room temperature for 36 h. The ethanolic extract solution (EEP) was then filtered through 0.45µm membrane filter and then the solvent was evaporated. The crude extracts were stored at -20°C until used. (Ertürk et al., 2016).

2.2. DNA Cleavage Assay

The test was applied according to the method previously described by Asmafiliz et al. (2013) and Ayvaz et al. (2018). Plasmid DNA pUC18 was used to examine the useful effect of the propolis extracts on hydroxyl radical-mediated DNA damage (Asmafiliz et al., 2013; Ayvaz et al., 2018).

2.3. Cell Culture and Virus

For virus infection, *S. frugiperda* cell line (Sf900) was employed as the host cell. Cells were prepared in Gibco Sf-900 medium complemented with 5% fetal bovine serum (FBS), 100 µg/mL streptomycin in 25 cm^2 culture flasks at 28 °C (Petcharawan et al., 2012). Through inoculating in Sf9 cells and incubation at 28 °C, AcMNPV stock was prepared. A week post infection, the virus suspension was transported and stock virus titer was investigated as TCID50/mL (tissue culture infectious dose per mL) and converted to pfu/mL (plaque forming unit per mL) (Petcharawan et al., 2012; Reed & Muench, 1938).

2.4. Cytotoxicity Assay

By using Sf9 cell line by MTT assay, different Propolis extract concentrations were analysed in terms of in vitro cytotoxicity (Mosmann, 1983). Crude propolis extracts were first dissolved in 1 mL of dimethyl sulfoxide (DMSO) and in order to get a stock solution of 6 mg/mL concentration, 10 mL volume was attained with protected medium, later diluted to different concentrations (200, 100, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL) in DMSO. On a microtiter plate reader, absorbance of the solution in each well in the plates was estimated at 570 nm.

2.5. Antiviral Assay of Propolis Extract at Different Concentration

Into each well of 24-well culture plates, $2.5x10^4$ cells in 5 % fetal bovine serum (FBS) added 0.5 mL Gibco Sf-900 medium were seeded and cultured for 4 h at 28 °C. To each well of 24-well plates, 6 mg/mL dissolved crude extract (diluted in unsupplemented Gibco Sf-900 medium, at concentrations of 200, 100, 25, 12.5, 6.25) was added and incubated for 1.5 h at room temperature for adsorption. Later, adsorption, the virus, and extract mixture were removed, phosphate buffer saline (PBS) was used to wash the cells and complete TNMFH medium containing 5 % FBS and 100 units/mL gentamicin, 100 µg/mL streptomycin medium solution was added to 24-well plates. 24 well culture plates were incubated at 28 °C for 4 days. When the period of incubation ended, the cells were first scraped and counted, and later in order to separate the the supernatant and the pellet, they were centrifuged at 4000 rpm for 20 min (Sökmen, 2001).

2.6. Statistical Analysis

For the estimation of cytotoxicity curves and CC50, GraphPad Prism 5.0 program was used. SPSS statistics 17.0 software was used to evaluate the data (Motulsky, 2007).

3. RESULTS and DISCUSSION

3.1. DNA Cleavage Assay

In this study, the DNA protection activity of propolis sample was investigated by using DNA interaction assay. Gel electrophoresis can be used to analyze DNA damage resulting from conformational change when H_2O_2 interact with pUC18 plasmid DNA. When the, the due to the can be investigated via agarose gel. Propolis extracts were dissolved in THF and pUC18 plasmid DNA was treated with different concentrations extracts, respectively. According to analysed gel electrophoresis results; Lane 1 and lane 2 were managed with untreated pUC18 plasmid DNA as a control, while lanes 3-6 pointed out plasmid DNA contacted with increasing

concentrations of the extracts in H_2O_2 condition (Figure 1). The results showed that propolis has a protective effect on DNA exposed with H_2O_2.

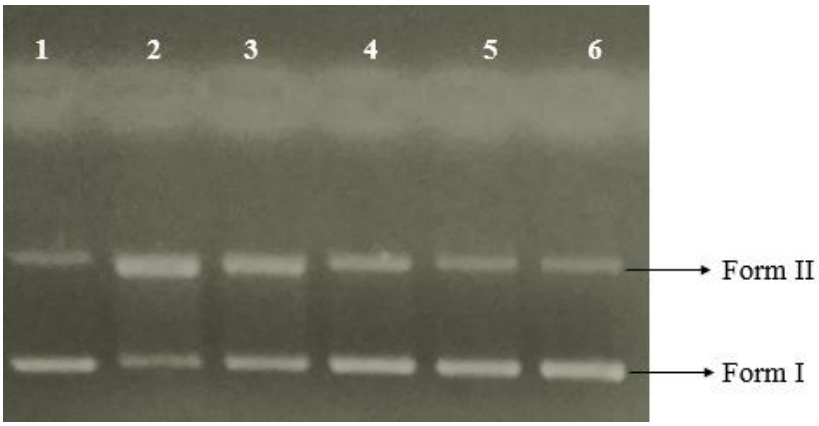

Figure 1. Gel electrophoretograms, Lane 1: control DNA; Lane 2: H_2O_2 and plasmid DNA; Lane 3-6: H_2O_2 + plasmid DNA and 12.5, 25, 50, 100 mg/mL extract

Plasmid DNA has three different forms as a supercoiled DNA form I, open circular form II and linear form III. These forms can be shown as the different lines on gel electrophoresis. Among these forms, the supercoiled form has faster mobility than other forms. In case of damage to one strand (nicking), as a result of relaxing to the open circular, the supercoil moves slower. In case of damage to both strands, linear form III appears between form I and form II (Akbaş et al., 2013; Asmafiliz et al., 2015).

3.2. Cytotoxicity Assay

Table 1 shows cytotoxicity assessment results of this concentration of propolis extracts against the Sf9 cell line. Based on the 60-hour exposure results with the prepared concentrations of propolis, at the concentrations tested (1.56 g / mL, 3.12 µg / mL, 6.25 µg / mL, 12.50 µg / mL, 25 µg / mL, 50 µg / mL, 100 µg / mL and 200 µg / mL), the decreases in cell viability were statistically significant compared to control ($p < 0.05$) (Table 1). It was also seen that the applied concentrations decreased cell viability depending on the dose (Figure 2). The concentrations of propolis have been shown to increase cytotoxicity due to dose increase (Figure 3) (Custódio, 2011; Gad 1999). Increases in cytotoxicity of all concentrations tested were statistically significant compared to control ($p<0.05$) (Table 1).

Table 1. Cell viability and cytotoxicity values calculated from data obtained as a result of 60-hour exposure of propolis concentrations

Dose	**%Cell viability**	**Cytotoxicity**
Control	100	0.00
1.56 µg/mL	0.14	99.86
3.12 µg/mL	-15.21	115.21
6.25 µg/mL	-22.72	122.72
12.5 µg/mL	-34.92	134.92
25 µg/mL	-43.81	143.81
50 µg/mL	-48.11	148.11
100 µg/mL	-48.31	148.31
200 µg/mL	-47.17	147.17

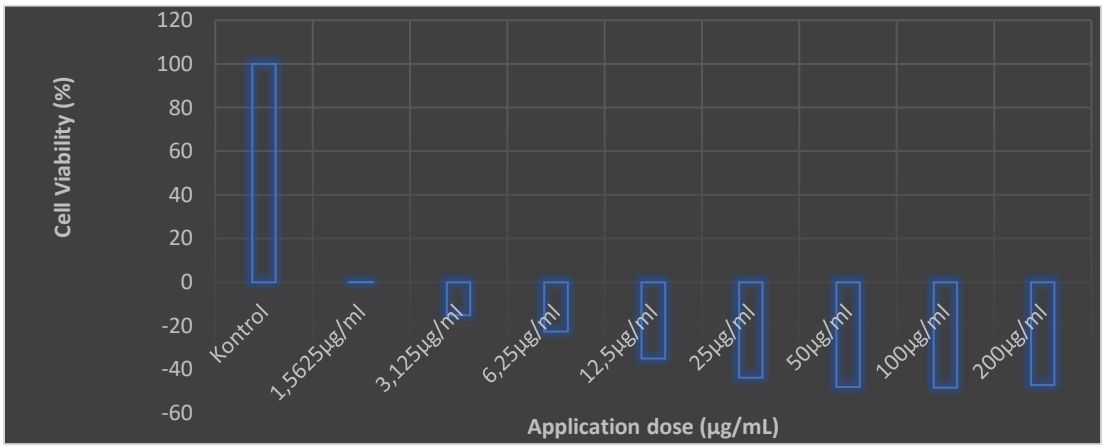

Figure 2. The effect of propolis on cell viability of cells after 60 hours of exposure Sf Cell (*: p<0.05)

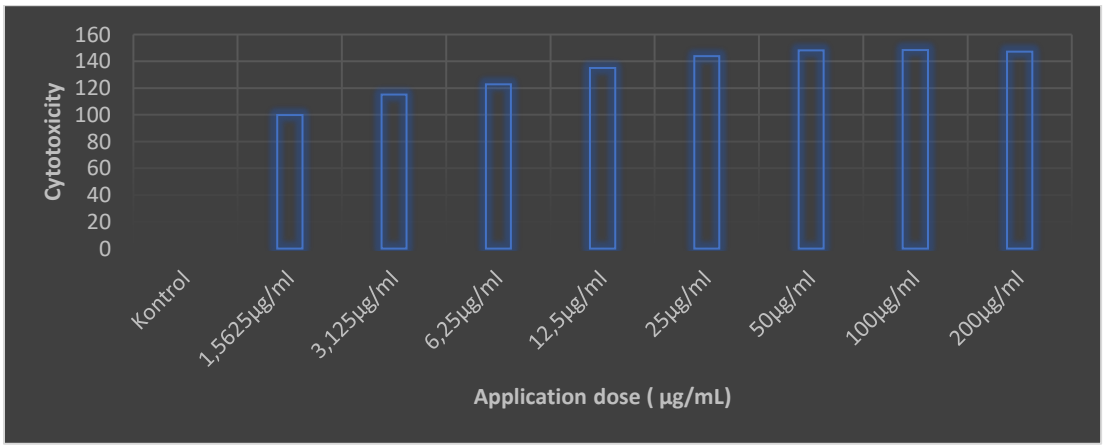

Figure 3. Effect of propolis on cytotoxicity of cells after 60 hours of exposure Sf cells (*: p<0.05)

3.3. Antiviral Activity

In the present study, according to antiviral activity result of ethanol extracts concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL against AcMNPV were shown in Table 2. In order to find out the antiviral activity of propolis extract on the replication of AcMNPV, all propolis extract concentrations were used. 1 h after contamination with AcMNPV, extracts were included at a multiplicity of infection of 1 and incubated at 28 °C for 60-72 h. Viruses were counted, especially when (OBs) were seen in the control group (Figure 4).

The IC_{50} value determined by logarithmic regression after 60 hours of propolis exposure was determined as 1.26 µg / mL (Table 2).

Table 2. The IC_{50} value obtained by treatment of cells with propolis.

Bee propolis	**Concentration** **60 h**
IC_{50}	1.26 µg/mL

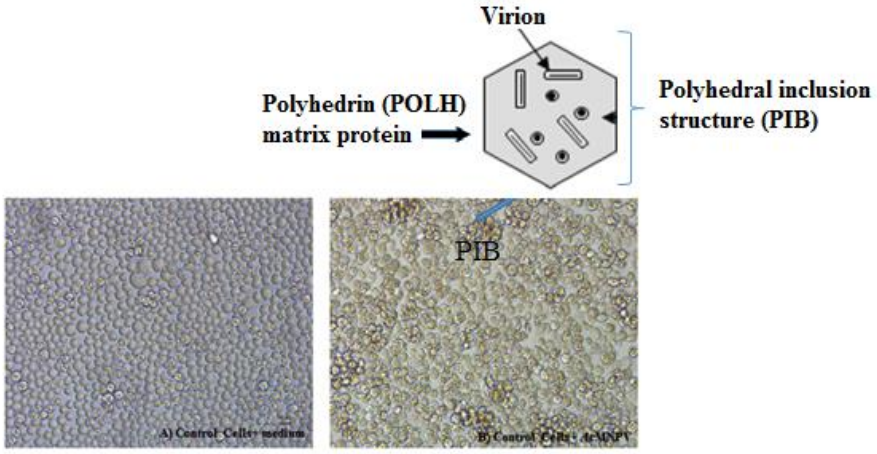

Figure 4. Phase-contrast micrographs of Sf9 cells (A), Sf9 cells after 60 h exposure in Gibco Sf-900 medium (B) Sf9 cells after 60 h post infection with AcMNPV Note, blue arrow shows polyhedral inclusion structure (PIB)

This process takes about 60 hours. Total number of occlusion bodies (OBs)/mL, the virus titter and the corrected percent reduction of virus titter were calculated. The results showed extremely important variation in the average virus titter between contaminated control cells (12.481) and contaminated cells in propolis extract of different concentration solutions (Figure 5).

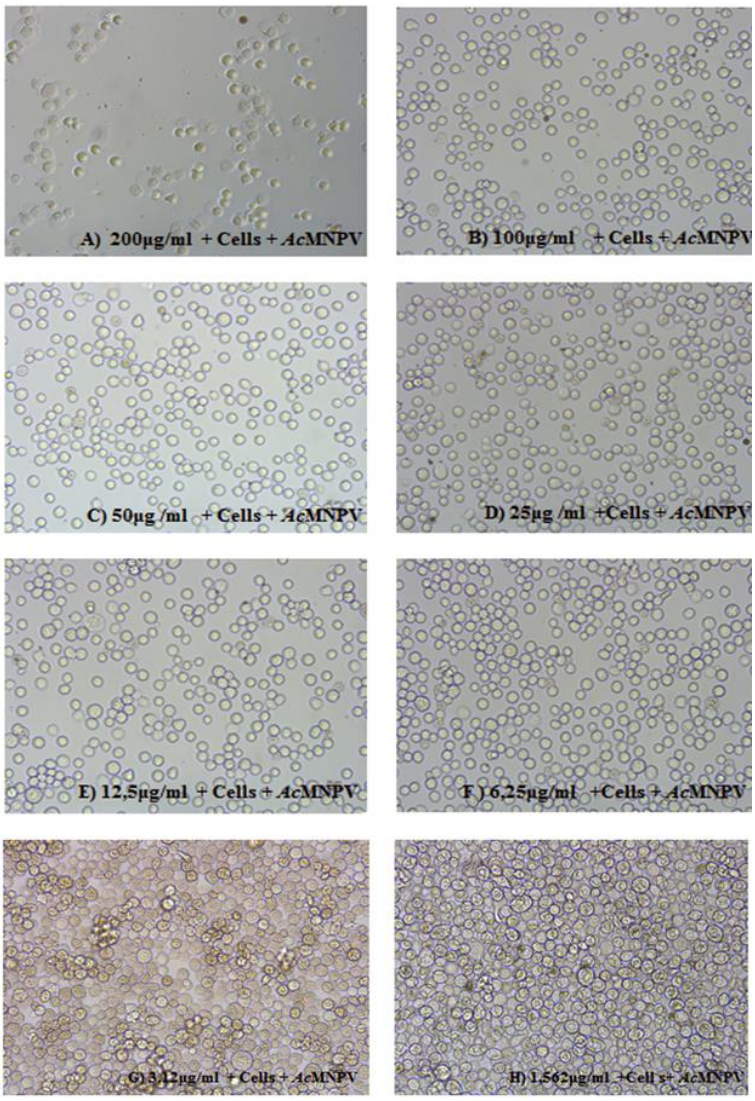

Figure 5. Phase-contrast micrographs of Sf9cells, Sf9 cells + various concentrations of propolis extract, A, B, C, D, E, F, H, G

When 1 h p.i was included in each concentration of propolis extract, virus concentration was found 1.586 pfu/mL for 100 µg/mL concentration of propolis extract and 1.601 pfu/mL 1.917 pfu/mL 2.570 pfu/mL 3.466 pfu/mL, for different concentration of propolis extract respectively. In terms of the production of occlusion bodies in Sf9 cells, no significant difference was found between the average percentage of contaminated cells in contaminated control cells when the concentration of propolis extract is 3.125, and 1.5625 µg/mL (4.018 pfu/mL and 5.145 pfu/mL respectively) ($p<0.05$) and no significant difference was found between the average number of occlusion bodies in the contaminated control cells and the contaminated cells in the concentration of propolis extract. Respectively 98.70% and 98.67% reduction were found in virus titter with propolis extracts when the concentration of propolis extracts 100, 50 µg/mL were included 1 h p.i. ($p<0.05$) (Table 3, Figure 6).

Table 3. Effects of propolis extract prepared in different concentrations on the offspring virus AcMNPV concentration

Cell + Different concentration of propolis extract Treatments	**Mean virus titer (PFU/mL)∗± S.D.**
Control Cells+ AcMNPV	$4.3x10^{9}$a ±5.42
200µg/mL propolis + Cells + AcMNPV	0
100µg/mL propolis + Cells + AcMNPV	$5.6x10^{7}$c±4.76
50µg /mL propolis + Cells + AcMNPV	$1.0x10^{8}$b±2.34
25µg /mL propolis +Cells + AcMNPV	$3.1x10^{8}$b±5.14
12.5µg/mL propolis + Cells + AcMNPV	$5.6x10^{8}$b±2.67
6.25µg/mL propolis +Cells + AcMNPV	$9.9x10^{8}$b±0.98
3.12µg/mL propolis + Cells + AcMNPV	$3.9x10^{9}$a±1.12
1.562µg/mL propolis+Cells+ AcMNPV	$3.9x10^{9}$a±4.42
Cell + Different concentration of propolis extract Treatments	**Mean virus titer (PFU/mL)∗± S.D.**
Control Cells+ AcMNPV	$4.3x10^{9}$a ±5.42
200µg/mL propolis + Cells + AcMNPV	0
100µg/mL propolis + Cells + AcMNPV	$5.6x10^{7}$c±4.76
50µg /mL propolis + Cells + AcMNPV	$1.0x10^{8}$b±2.34
25µg /mL propolis +Cells + AcMNPV	$3.1x10^{8}$b±5.14
12.5µg/mL propolis + Cells + AcMNPV	$5.6x10^{8}$b±2.67
6.25µg/mL propolis +Cells + AcMNPV	$9.9x10^{8}$b±0.98
3.12µg/mL propolis + Cells + AcMNPV	$3.9x10^{9}$a±1.12
1.562µg/mL propolis+Cells+ AcMNPV	$3.9x10^{9}$a±4.42

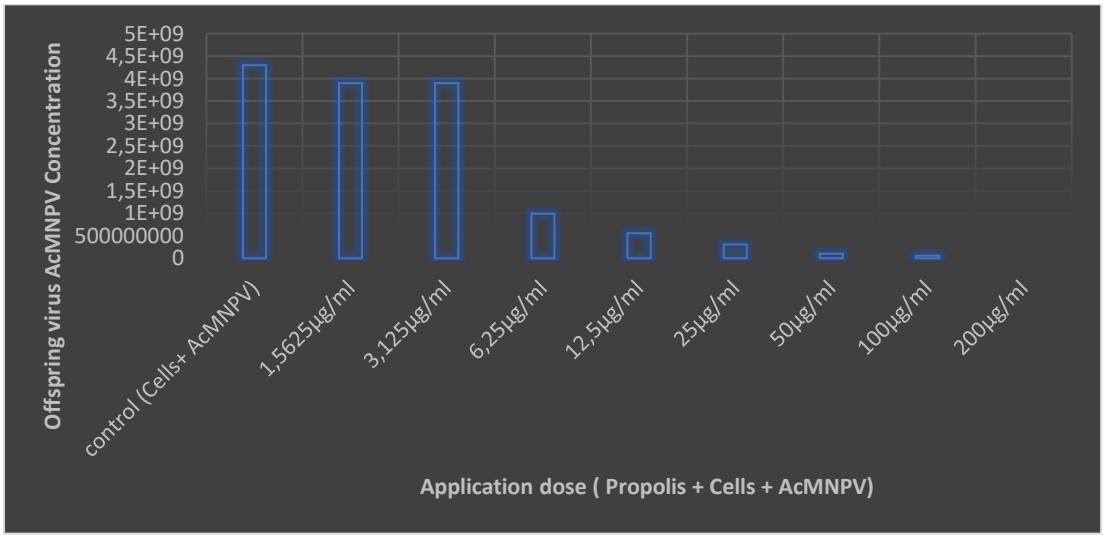

Figure 6. Effects of propolis extract prepared in different concentrations on the offspring virus AcMNPV concentration

The results showed that in Sf9 cell line, DNA replication of AcMNPV was restricted and antiviral influence was found in ethanol extract of propolis. Antiviral influence of extract from crude hexane extracts of *A. cathartica* plant was analyzed by Petcharawan et al. (2012). They also stated that higher antiviral effect was found from leaf extracts and virus concentration decreased by 66%. Humans are not infected by baculovirus; however, replication cycle of AcMNPV is similar to double-stranded DNA viruses causing infection in people. 100, 50, 25 µg/mL concentrations of propolis extracts were not found to influence the growth of Sf9 cells, just as 6.25, 3.125, and 1.5625 µg/mL concentrations. These concentrations were not found to have restricting effects on the replication of AcMNPV in Sf9 cell line, although extracts of the same concentrations were found to have a high significant restrictive effect on the replication of AcMNPV. Respectively 98.70 % and 98.67 % decrease were found in the virus concentration when 1 h p.i (post infection) was added in the stem and leaf extracts (Ertürk et al., 2000; Oktar, 2009). The results showed that when different concentrations of propolis extracts were applied, replication steps of the virus were restricted. As a result, while the replication of the virus was hindered, it was possible for the cells to continue their normal growth and proliferation (Vanhaelen & Vanhaelen-Fostre, 1979). It might be possible to explain the properties of propolis with high content of flavonoids. It was shown that quercetin and luteolin are virucidal. Viruses which were sensitive were only those that had membranes (Kaul et al., 1985). For this reason, flavonoids present in propolis might be the cause of extracellular inactivation of enveloped viruses. As shown by this study, some other studies have also shown that targets of inhibitory actions are synthesis of DNA, and RNA and virus-specific enzymes including thymidine kinase, DNA polymerase, deoxyribonuclease, and ribonucleotide reductase (Poncede et al., 1997).

4. CONCLUSION

The virus AcNPV used in the study is quite important because it is a good model for other pathogenic DNA viruses such as herpesvirus, adenovirus and vaccinia virus of humans. Since this is a screening study, it is better to utilize a nonpathogenic virus rather than pathogenic viruses of humans. Once any effective concentrations of propolis extract is determined, this substance can be purified and used on pathogenic DNA viruses. On the other hand, the concentrations of propolis extract, which has a reducing effect on virus replications, and the fact that it contains different compounds especially for the control of viral diseases, also provides the opportunity to use these compounds as raw materials in the pharmaceutical industry by purifying them.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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