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RESEARCH PAPER

Comparative Study of Some Commercial Propolis Extract with New Prepared Ethanolic Propolis Extract

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Abstract

In this study, quality parameters of some commercially sold in Turkey propolis extracts were compared with freshly prepared ethanolic propolis extract (70%). The pH, total phenolic substance, total flavonoid and total antioxidant capacity values of 11 different commercial samples, including aqueous, ethanolic, water-based and vegetable oil, were compared. As a result, it was determined that ethanolic samples had the highest activity and the activity of propolis with olive oil was very low. It was found that products with high total polyphenol content (TP) had high total antioxidant values. It was also determined that a standard method was not used for the preparation of commercial propolis samples and that the TP values differ greatly.

Introduction

Honey, pollen, propolis and royal jelly are the most used bee product in apitherapic applications (Sahin & Kemal, 2020). Propolis is a natural paste as a result of the accumulation of resinous substances collected from the bark, leaves and stems of the honey bees in the hives. This complex mixture, which is used to protect the hive for multiple purposes, is an indispensable natural bee product of apitherapy (Akçay, Birinci, Birinci, & Kolaylı, 2020; Bankova et al., 2019). It is known that the best solvent for raw propolis is 70% ethanol, but some glycol derivatives and vegetable oils are also usedin the preparation of extracts (González-Búrquez et al., 2018; Oroian, Ursachi, & Dranca, 2020). The apitherapy active ingredients of propolis are a family of polyphenols. It has subclasses such as polyphenols, phenolic acids, flavonoids, anthocyanins and tannins, which have a wide range of compounds. Phenolic acids are more hydrophilic, that is, bimolecular dissolving in aqueous environments and flavonoids in hydrophobic environments. For this reason, flavonoids are insoluble in aqueous media, and phenolic acids such as gallic acid in apolar solvents (Galeotti, Maccari, Fachini, & Volpi, 2018). Propolis is one of the natural products with the highest biological activity and is a moist agent of traditional and complementary medicine with its high antioxidant, antimicrobial, anti-inflammatory and antitumoral properties (Kolaylı & Keskin, 2020; El Adaouia Taleb, Djebli, Chenini, Sahin, & Kolayli, 2020). Until now, an ideal solvent for the raw propolis has not been found exactly. However, since ethanol is more apolar than water and more polar than oils, it has the ability to dissolve a large number of both polar and apolar compounds.

The aim of this planned is to investigate whether commercial propolis extracts are in the order of total polyphenol and other quality.

Materials and Methods

Samples

11 commercial propolis samples were purchased from various markets and herbalists. A codename data for each sample was not used in stealing the trade name. The crude propolis specimen was made in Turkey, named also Anatolia propolis. Fifteen different regions of propolis sample (10 g) of Anatolia were collected obtained a pool. 10 g of powder sample was taken the pool and extracted in 70% ethanol (1:10 w/v). It was protected in the refrigerator at + 4 ºC.

Extraction

For the extraction, it was first extracted in an ultrasonic bath for 1 hour and then in a shaker (Heidolph Promax 2020, Schwabach, Germany), for 24 hours. Then it was filtered and used in the study. Water-based and olive oil samples were prepared by extracting them in 70% ethanol for 24 hours**.**

Total Phenolic Content

The total amount of phenolic substance was measured by Folin Ciocalteu method (Singleton & Rossi, 1965). This method contains all the polyphenols in solution and reflects their total values. 10 mL of different concentrations of standard and 10 mL ethanolic samples (1 mg/mL), 200 mL of 0.2 N Folin–Ciocalteu's regents and 680 mL of distilled water were mixed. Following 4 min incubation, 400 mL of Na_2CO_3 (10%) was added. The mixture was incubated for 2 h then absorbance was read at 760 nm. The calibration curves were occurred using each individual standard at six different concentrations (0.5; 0.25; 0.125; 0.0625, 0.03125 and 0.015 mg/mL) and all analysis were carried out in triplicate. Results were expressed as milligrams of gallic acid equivalents per 100 mL sample (mg GAE 100 g/mL) by using a standard graph.

Total Flavonoid Determination

Determination of total flavonoid substance was used according to Fukumoto and Mazza (2000). It was used quercetin in different concentrations (QU) for standard. Total flavonoid amount as quercetin equivalent as mg quercetin (KE)/100 g honey. The total amount of flavonoid substance was found by measuring the absorbance of the coloured product formed as a result of the redox reaction between flavonoids and aluminium (III) at a wavelength of 415 nm. Working solutions of 0.500, 0.250, 0.125, 0.062, 0.031 and 0.015 mg/mL were prepared from this stock by serial dilution and the absorbance values were read according to the method. A standard calibration graph was obtained by drawing a graph of absorbance against quercetin (1 mg/mL) concentration. Using the standard calibration graph, the total amount of flavonoids in the sample was calculated and the results were given as mg Quercetin equivalent per gram sample (mg QE/mL).

Total Antioxidant Activity

The ferric reducing antioxidant power (FRAP) was used to measure the total antioxidant power of the samples (Benzie & Strain, 1996). Firstly, FRAP reagent was prepared with 25 mL of 0.30 M acetate buffer (pH 3.6), 2.5 mM TPTZ solution in 40 mM hydrochloric acid, and 2.5 mM $FeCl_3$. $6H_2O$ solution. Briefly, 100 mL of standard or samples solution was added to 3 mL of freshly prepared FRAP reagent and the reaction mixture was incubated at 37 °C for 4 min. Then, the absorbance was measured at 593 nm against distilled water as blank by using the spectrophotometer. Trolox was prepared at different concentrations (31.5, 62.5, 125.0, 250.0, 500.0 and 1000 mM), using as a standard antioxidant compound. The results were expressed in micromole Trolox equivalents per gram.

Results and Discussion

Table I summarizes the quality parameters of some trade propolis samples from Turkey. And also, the pH values of all propolis extracts were compared. It was determined that the pH values varied between 4.0 and 5.0 except for only 2 samples. The pH values of ethanol and glycol derived samples were found to be around 4-5 acidic. The pH was 9 in one aqueous sample and 10 in the other. It is normally crude phenolic acids, and when dissolved in water or alcohol, it is below pH 6. The pH of the various phenolic acids found in propolis is below 7. Interestingly, it was considered quite surprising that the pH of the two samples was well above 7. The exact reason for this is unknown, but it is thought that it is not from crude propolis and that a basic solvent is used to dissolve the propolis. While the stomach pH varies between 1.5-2.0, the pH of the foods we consume is around 4-5. Direct consumption of propolis extract at such high pH can cause stomach damage. Therefore, the pH should be taken into account when standardizing propolis extracts (González-Búrquez et al., 2018; Galeotti et al., 2018; Bankova et al. 2019).

The most important feature of propolis extracts are polyphenols in their structure. Total polyphenol content (TP) is the most important determinant for propolis, and high polyphenol value indicates high biological activity. Table I gives the total polyphenol values. Accordingly, to the result, the total polyphenol values of ethanolic, aqueous and glycol extracts differ. It was determined that the highest TP value was in ethanolic samples, and the water and olive oil samples had the lowest activity.

Table 1. Comparative study of some commercial propolis extracts of Turkey.

Code	Used Solvent	pH	Total phenolic	Total Flavanoid	FRAP
Anatolia Propolis					
P	Ethanol	5.30	5105±120	2040±23	285±32
Ethanolic commercial extracts					
P1	Ethanol	9.0	1093±34	752±21	266±17
P3	Ethanol	5.12	5931±93	1945±12	858±74
P4	Ethanol	5.10	3287±21	2114±39	553±22
P5	Ethanol	4.80	2605±34	1044±24	687±11
Water-Soluble commercial extracts (glycol derivates)					
P6	Glycol	5.20	2283±42	2116±22	363±85
P7	Glycol	5.30	3090±23	2040±21	320±53
Water commercial extracts					
P8	Water	10.00	645±23	449±37	58±80
P9	Water	5.40	102±4	17±1	33±4
Olive oil commercial extracts					
P10	Olive oil	4.00	492±7	260±8	98±1
P11	Olive oil	4.20	642±16	310±22	246±9

Total phenolic: mg GAE/100 mL,
Total Flavanoid: mg QE/100 mL,
FRAP: mmol $FeSO_4 7H_2O$/mL

It is seen that the TP value of ethanolic extracts varied between 1093 and 5931 mg GAE/100 g. Samples dissolved in glycol were found to have lower TP than ethanol, but significantly higher than aqueous and olive oil samples. While there are significant differences between the two water-soluble samples and the differences is too high. The reason why the water-based P8 sample, which is soluble in water at any ratio and has a very dark red colour, contains approximately 10 times higher than the P9 sample. However, when this sample is evaluated together with the high pH value, it is thought that this may be due to the solution used. It was found that oil-based propolis extracts had values close to each other. Comparing the TP values of commercial propolis samples with Anatolian propolis that we prepared in the laboratory, only one ethanolic sample contained higher polyphenols and the others were found to be lower.

When the total flavanoid amount (TF) was examined, it was found that ethanolic samples had the highest flavanoid value. In the study, it was found that TF values increased or decreased in parallel with TP values and the lowest TF values were in aqueous samples.

Total antioxidant capacity (TAC) values were calculated in terms of the reducing ability of the Fe (III) complex in the study. While high FRAP value indicates high capacity, it was determined that the highest FRAP value was in the commercial ethanolic sample and it was followed by Anatolian propolis. Olive oil samples and water samples were found to have very low TAC values.

When studies on propolis are scanned in the literature, studies with mostly raw propolis draw attention. Since there were not many studies on commercial propolis, there was no possibility of comparison or discussion.

Conclusion

As a result, the amount of solvent and solvent used in the extraction of raw propolis is very important. However, the most important criteria to be sought in commercially purchased propolis are the use of solvents that will not harm human health and that products with high polyphenol content are more valuable.

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RESEARCH PAPER

Effect of Number of Worker Bees in Queen Mating Nucleus on Queen Quality and Mating Nucleus Population Dynamics

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Abstract

This study aims to investigate the effects of the amount of worker bees placed into the mating nucleus on queen quality parameters and nucleus population dynamics. The quantity of 80(g) (~700 workers) that mating nucleus made of Kirchhain-model can contain has been based on. A total of 24 mating hives, including 8 in each group containing 80, 160 and 320 grams worker bees increasing at a geometric progression, were used in the study. Each mating nuclei been used in queen production for 3 times (20 day interval) without any addition of worker bees, depending on the climate and vegetation conditions during the production period. In groups containing 80,160 and 320 gram workerbees, spermatheca diameters (mm) 1.14±0.19, 1.11±0.13 and 1.16±0.09, sperm counts stored in spermatheca 3268859±288.830, 4217444±182468 and 4875752±304443, live weights of the laying queen (mg) 188.5±2.55, 205.8±4.09 and 229.6±4.34, worker bee weights (g) in mating nuclei after research were 100.85±4.39, 223.85±14.44 and 413.66±43.84 found to be respectively. At the end of the study conducted in Hatay Province between April 25 - June 25 2019, overall increase in the worker bee population was observed average to be 31.87±5.39%. The population increase in 80, 160 and 320 g of live worker bee groups were observed to be 26.07±5.47, 39.91±9.02 and 29.27±13.70% respectively. After 60 days & 3 periods of queen breeding the differences between groups were found to be statistically insignificant (P>0.05). The differences between the groups, the sperm counts, laying queen weight and amount of worker bees were found to be statistically significant (P<0.01).

Introduction

Beekeeping is an archaic agricultural activity being performed in Anatolia since ancient times. Factors such as topographic and climatic characteristics of Turkey, it's status of natural bridge between Africa, Europe and Asia, the presence of many honey bee races and ecotypes in its fauna, and its rich floristic and faunistic biodiversity have allowed apiculture to be a sustainable agricultural activity in the region for thousands of years.

It has been known that local honey bee ecotypes such as Muğla, Yığılca, Gokceada, etc. are present in Turkey, as well as 5 (*A. m. anatoliaca, caucasica, syriaca, carnica* and *meda*) of 26 honey bee races identified in the world. Local honey bee races and ecotypes in Turkey have survived for tens of thousands of years by adapting to biotic and abiotic conditions in their respective regions. Although Turkey possesses great genetic diversity in terms of honey bee race and ecotypes, the apiculture sector could not benefit sufficiently from the genetic diversity Turkey possesses. In addition, breeding methods could not be developed to obtain local races and ecotypes in terms of economically substantial physiological and behavioural traits. Therefore, sustainable pure breeding race, line and commercial use hybrids could not be generated from them. The sector has had to make commercial production due to the presence of unproductive hybrids of races and ecotypes that copulate with each other in an uncontrolled manner as a result of improper queen production, method and policies. Use of them in intra-regional and inter-regional migratory beekeeping practices by marketing local genotypes adapted to certain habitats to eco-geographic regions very different from their

natural habitats have caused genetic pollution across the country.

Physiological and behavioural traits of honey bee colonies are related to breeding value and quality of the queens used and the genotype and number of drones with which they mate, as well as to beekeeping practices (colony management, feeding, etc.) and their habitat and climate conditions. In the majority of commercial queen-rearing enterprises with a production permit, breeding bee selection is made according to body color, but drone production and breeding have been hardly ever made.

According to the year 2020 data of the Ministry of Agriculture and Forestry, there are 8128360 honey bee colonies in 80675 beekeeping enterprises in Turkey. During the 2019 beekeeping period, 109330 tons honey were produced (Anonymous, 2020a). For sustainable economic beekeeping, it is necessary to change the queens of colonies used in commercial production yearly if possible, otherwise biyearly. Considering the additional queen losses in honey harvest, colony increases and beekeeping practices during the production period, the sector needs at least 4-5 million of pure race/ecotype, breeding and commercial use hybrid quality queens yearly. It is inevitable to experience yield and colony losses in beekeeping practices devoid of a queen replacement system.

Although it varies according to years in Turkey, there are 146 commercial queen production enterprises that can operate with a total production capacity of 510200 / year and have received production permit according to the 2020 data of the Ministry of Agriculture and Forestry. There are 6 breeding and 7 licensed queen production enterprises owned by the public and private sector where the queens can be used for breeding purposes. There is a total of 12 races/ecotypes registered under the names of “Anatolian Ecotype, Central Anatolian Ecotype, Caucasian Ecotype, Thrace Ecotype, Aegean Ecotype, West Aegean Ecotype, R&D, Ecotype, Giresun Ecotype, Muğla Ecotype, Black Sea Ecotype and Yığılca Ecotype” in these enterprises (Anonymous, 2020b).

Since the shortage of approximately 4 million of queen per year the sector needs cannot be compensated by commercial queen production enterprises, this causes an increase in the rate of old and poor-quality queen use and an increase cannot occur in the desired efficiency. The beekeepers mostly use swarm queens and queen cells in re-queening, colony division and production facilities. The colonies generated by swarm queen and queen cells, bad selection is made that increases the frequency of swarming behaviour in future generations, which in turn causes unwanted physiological and behavioural traits to continue increasingly.

Queens are the most important individuals in terms of their position in the colony. By combining the hereditary traits they get from the drones during the mating flight with their own hereditary characteristics, the queens transfer them to the worker bees with different numbers and degrees of kinship in the superfamily structure that forms the colony. While the drones mating with the queens dies, the sperms of the dying drones can survive for as long as 5 years in the spermatheca of the queen in a mixture with the sperms of other mating drones.

Nuclear colonies queenless in different physical structures and sizes are used in commercial queen production, which are called the mating nucleus, where the queen pupae come out of the cells and perform the first offspring raising activity by mating. During the preparation of the mating nucleus, different numbers of worker bees are placed for the care and feeding of the queens according to their physical characteristics, and the worker bees in each box are used for the mating with more than one queen sequentially during the production season. The number and physiological age composition of worker bees in the mating nucleus vary depending on time. New worker bees, which can be generated from the eggs of young queens, can extend the effective use of mating nucleus during the production season. This study aims to investigate the effects of the amount of worker bees placed in a queen mating nucleus on thequeen quality and mating nucleus population dynamics in the oncoming production process.

Materials and Methods

The research was carried out in the Eastern Mediterranean coastal zone (Hatay Province), which is one of the locations where seasonal climatic conditions and vegetation become suitable for queen bee breeding at the earliest period in Turkey. The research was realized at 36°19'49.60 "North latitude, 36°11'33.29" East longitude an altitude of 145 m from the sea between 25 April-25 June 2019, at Mustafa Kemal University Agricultural Research and Application Center.

Establishment of Mating Nucleus

A total of twenty-four mating nuclei were used in this study. Eight nuclei were made high-density polystyrene Kirchhain model and remains sixteen nuclei had 5 standard full depth Langstroth timber-framed wooden mating boxes. The nuclei were filled up with young worker bees by shaking from combs of brood nest taken from Anatolian honey bee (*Apis mellifera anatoliaca*) colonies two days ago before introducing the queen cells. The shaken young bees were anaesthetized with CO_2 and weighted analytical balance with 0.01 mg precision before the installation to nuclei. Nuclei were categorized into three groups contain eight nucleus. Each groups filled with 80 g, 160 g and 320 g worker bees.

Table 1. Post-mating average Live Weights (± S.E.) (mg) and percent population change of queen breeding in nucleus with different quantity of worker bees.

Amount of Worker Bee (g)	n	Mated ♀ Weight (mg) $(\overline{X} \pm S\bar{x})$	n	Population Increase (%) $(\overline{X} \pm S\bar{x})$
80	23	188.5±2.55 a	7	26.07±5.47
160	21	205.8±4.09 b	7	39.91±9.02
320	19	229.6±4.34 c	6	29.27±13.70
Total	63	206.3±2.96	20	31.87±5.39

[a,b]: Averages with different superscript denoted by small letters in the same column are significantly different (P<0.01).
Duncan - Harmonic average sample size = 20.456

Regulating the Number of Mature Bees In Nuclei

In production season the anaesthetized with CO_2 50 grams of worker bees were counted and the average adult worker bee weight was calculated as 115 mg. The average surface area (cm^2) of a worker bee on the comb was calculated by measuring the width and length of 100 workers. As a result of the calculation, it has been estimated that the average one worker bee occupies 1.152 cm^2(1.6 cm × 0.72 cm) areas on the honeycomb surface. The number and weight of adult worker bees were calculated based on the number of bees that could fully cover the total of 800 cm^2 honeycomb surface area in the Kirchhain model breeding box. Honeycomb surface area (800 cm^2) were divided one worker surface area (1.152 cm^2) and found approximately 700 worker bees. Worker number multiplied with average adult worker bee weight (115 mg) approximately equal 80 g live weight of worker bee. The other two research groups are planned to contain 160 and 320 grams of worker bees, increasing in geometric progression.

Wooden mating nuclei were arranged for one empty honeycomb, one foundation frame and one high-density polyurethane division board for insulation.

In this study, Anatolian honey bee (*Apis mellifera anatoliaca*) colonies were used for breeding. One day old larvae were grafted into beeswax queen cell cups by the Doolittle method and were given to starter colonies prepared queenless. In addition to similar applications such as proportional feeding, same colonies were used to eliminate the effects of differences between genotype, starter and finishers.

Mature queen cells were randomly introduced into mating nuclei in the period of ten days after grafting. All queens emerged from the queen cells on the second day after the cells were introduced into the mating nuclei. In order to identify the emergence of queens, mating nuclei were inspected regularly. For the continuity of the mating boxes, the transition of the larvae to the prepupa period (capping brood) was expected. Following, mated queens were collected from nuclei. The mated queens were weighed using an analytical balance with 0.001 mg precision and subjected to dissection to remove spermatheca.

The tracheal net around the spermatheca was cleaned gently and carefully by using fine insect needle and forceps. The diameter of spermathecae was measured under a stereomicroscope equipped with an ocular micrometer. Spermathecae diameters were calculated in two distant points and the average of two measurements were taken. The average radius of each spermatheca was then used to calculate the spherical volume formula: $4/3\ \pi r^3$

The spermathecae were then discharged with a fine forceps in 1 ml of saline solution (0.9%) in a concave watch glass dish. The final volume was completed by 4 mL tap water adding to 5 mL. Spermatozoa numbers in spermatheca of the queens were counted five replicates by using a Thoma counting chamber under a light microscope (Mackensen & Tucker, 1970).

All the virgin queens were mated naturally without drone bee production in research apiary. At the end of queen production season, the workers in nuclei anaesthetized with CO_2were weighted with an analytical balance of 0.01 mg precision. The total live weight change of worker bees between the beginning and the end of the study was determined as weight and percentage.

Statistical Analyses

Randomized plot design (ANOVA) was used to test the diameter of spermatheca, the number of spermatozoa in the spermathecae, spermatheca volume, live weight change of worker bees and queen weight. Levene's homogeneity test were used for equality of variances. Duncan's Multiple Range Test was used to compare the means between groups. Mating ratios and acceptance rates were analyzed using Chi Square non-parametric tests (Cooley & Lohnes, 1971). Linear Regression analysis was applied to determine the correlations between dependent and independent variables (Little & Hills, 1978). Correlation coefficients between all measured character pairs were also calculated. All statistical analyses were performed using SPSS v. 23 for Windows.

Results

This research was initiated at the Apiculture and Silkworm Application and Research Center located on the Tayfur Sökmen Campus of Mustafa Kemal University on 25 April 2019. Since abnormal temperature increase is observed depending on global climate changes in June, all of the research materials have relocated to a higher altitude area with dense leaves-shady trees within that region (9-25 June). Only three generations/periods of the queen could be bred in the region during the research lasting for two months.

During the research, absconding behaviour was not observed in any of a total of 2 types-24 mating nucleus containing 80, 160 and 320 grams of live worker bees. Queen was occurred to emerge from all mature queen cell transferred to the mating nucleus in all groups. Two mating nucleuses were omitted from the experiment because wing deformation was observed in queens emerging from the one for each cell in groups containing 160 and 320 grams of worker bees from mature queen cells placed in the mating nucleus by producing from the first group larva transfer.

As wing deformation was observed in one queen that emerged from the cell in the third production in the group containing 80 grams of worker bees, one nucleus from each group (a total of 3) was excluded from the experiment throughout the research. Since one queen could not return from its mating flight in the first graft in the group containing 320 grams of worker bees, the relevant nucleus was excluded from the experiment. Therefore, differences appeared in the number of subgroups.

Table 1 presents the post-mating average live weights (mg) of the queens being bred in nucleuses containing 80, 160 and 320 g of live worker bees and the percent population changes (%) in the nucleuses at the end of the experiment. Post-mating live weights of the queen were 188.5±2.55, 205.8±4.09 and 229.6±4.34 mg, respectively. The differences between average weights of the queen were statistically significant (P<0.01, df: 2, F: 31, 261) according to the ANOVA test.

The average weight of mated queen in the study groups was calculated as 206.3±2.96 mg. At the end of the research, it was observed that the increase in the amount of worker bees in the nucleus where the queens mate significantly increased the weight of the mated queen.

After 3 periods of queen breeding within 60 days, the average weights of the worker bee in the nucleus containing 80, 160 and 320 grams of worker bees were found to be 100.85 ± 4.39, 223.85 ± 14.44 and 413.66±43.84 g, respectively. When percent weight changes at the end of the research were calculated based on the initial weights, an average population increase was 26.07%±5.47, 39.91%±9.02 and 29.27%±13.70 respectively, between the groups. Overall, an average population increase of worker bee was 31.87%±5.39 in all groups. Although the highest increase was observed in 160 gr of worker bee group, there were statistically insignificant differences in percent worker bee population between group averages (P>0.05, sd: 2, F: 0.60).

The average diameter of spermatheca (mm) and the number of spermatozoa (piece) determined in queens bred in nucleus containing 80, 160 and 320 grams of live worker bees are given in Table 2.

Spermatheca diameters of 80, 160 and 320 g of worker bee groups were 1.14±0.19, 1.11±0.13 and 1.16±0.09 mm, and the differences were statistically insignificant between group averages (P>0.05, sd: 2, F: 2.17). The number of spermatozoa stored in spermatheca was 3268859±288830, 4217444±182468 and 4875752±304443 respectively. The differences were statistically significant between the group averages of the number of spermatozoa stored in the spermatheca (P<0.01, sd: 2, F: 9.383). At the end of the research, it has been observed that the population size of the worker bee in the queen bee mating nucleus affected positively the number of spermatozoa stored in the spermatheca.

The differences were statistically insignificant (P>0.05) between the average spermatheca diameters (mm) of the mating queens in the research groups, while the differences were statistically significant between the average values of spermatheca volume (mm^3) calculated mathematically on the sphere formula (P<0.05, sd: 2, F: 3.417)(Table 3).

At the end of the research, it was found that the queens bred in queen mating nucleus with 320 g of worker bee population size had the highest and most stable values, with a 0.794±0.009 mm^3 of spermatheca volume value.

Discussion

A study on "Queen Production Costs and Profitability Analysis in Turkey" by Karaca and Karaman (2018) reported that, during the queen production period, the rate of commercial producers making queen production three times without interruption in a mating nucleus was 75%, the rate of commercial producers making queen production four times was 14% and the sales period of the first queen started in the second week of April.

Yeninar, Akyol, and Yörük (2007), found that pre-sale live weights of a total of 2.283 mated queens, which they put on the market in plastic queen cages by 7 different commercial queen production firms between April 20 and June 09, 2005, were 189.96±0.5 mg on average within the range of 174.2 to 207.2 mg.

Arslan, Arslan, Cengiz, and Karakuş (2018) found that a total of 105 queen samples raised in 21 commercial producers in the early period in the Mediterranean coastal region had an average of 191.04±2.094 mg live weight (± SE), had an average of

Table 2. Average spermatheca diameter (± S.E.) (mm) and spermatozoa number (pieces) of queen breeding in nucleus with different quantity of worker bees.

Amount of Worker Bee (g)	n	Spermatheca Diameter (mm) $(\overline{X} \pm S\bar{x})$	Spermatozoa Number (pieces) $(\overline{X} \pm S\bar{x})$
80	23	1.143±0.19	26.07±5.47
160	21	1.116±0.13	39.91±9.02
320	19	1.163±0.09	29.27±13.70
Total	63	1.140±0.09	31.87±5.39

[a,b]: Averages with different superscript denoted by small letters in the same column are significantly different (*P*<0.01).
Duncan - Harmonic average sample size = 20.456

Table 3. Post-mating calculated spermatheca volume (mm^3) of queen breeding in nucleus with different quantity of worker bees.

Amount of Worker Bee (g)	n	Spermatheca Volume (mm^3) $(\overline{X} \pm S\bar{x})$	Minimum	Maximum
80	23	0.742±0.026[ab]	0.51	0.98
160	21	0.726±0.116[a]	0.63	0.84
320	18	0.794±0.009[b]	0.74	0.88
Total	62	0.752±0.011		

[a,b]: Averages with different superscript denoted by small letters in the same column are significantly different (*P*<0.05).
Duncan - Harmonic average sample size = 20.456

1.044±0.071 mm spermatheca diameter, had an average of 0.605±0.012 mm^3 spermatheca volume and had 4.454±0.177 million spermatozoa.

In a study carried out in Hatay, Uzun (2020) reported that post-mating live weight of the queen was 186.76±2.87 mg, the spermatheca diameter was 1.18±1.09 mm, the spermatheca volume was 0.87±0.02 mm^3, and the number of spermatozoa present in the spermatheca was 4655369±428171 per queen.

The data from this study are consistent with the quality parameters from commercial and research queen produced at equivalent queen production dates in different years in the Mediterranean coastal zone.

Conclusion

The first queen production of the year in Turkey has being made in the Mediterranean coastal region where climate and habitat are the most suitable. Many producers engaged in migratory professional beekeeping activities also prefer the same region for overwintering. Demand for the massive queen after overwintering in the region is seen in April and May, the months of which coincide with the natural swarming season when pollen and nectar sources are most abundant in the relevant region. The climate and habitat characteristics of that region have a stimulating effect on quality queen production. Due to the reasons mentioned above, almost all of the commercial queen producers overwinter in the Mediterranean coastal region and market their first productions to beekeepers before the highland honey production period.

Intensive intra- and interzonal migratory beekeeping activities in Turkey cause queens to rapidly burn out and age in physiological terms. Due to old and physiologically burnt-out queen colonies, the colony population cannot reach a sufficient level during the production season, and significant winter losses are experienced depending on the negative effects of global climate changes.

An average of 40 mg live weight increase (229.6±4.34 mg) was observed in a statistically significant manner (P<0.01) between queens raised in Kischain model-nucleus with 80 g of worker bees and those raised in standard Langstroth framed-nucleus with 320 g of worker bees. Overall, the live weight of young queens used in production should be 200 mg and above (Akyol, Yeninar & Kaftanoglu, 2008) since this is

considered a quality criterion. Population development, acceptance rate of young queens and production efficiency are higher in colonies of queen with a great live weight. Similarly, an increase of spermatozoa was detected in a statistically significant manner (P<0.01) by an approximate rate of 50% in the spermatheca of queens. These results affect positively the effective use of queen in production during commercial beekeeping activities. In addition, they can be used easily in production and adult bee trade with standard beekeeping materials by renewing worker bees in standard Langstroth framed-nucleus after the queen production period. Therefore, sustainable production can be achieved. Addressing this current issue economically in other researches would contribute to the apiculture sector.

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RESEARCH PAPER

The Effects of Some Essential Oils Against Nosemosis

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Abstract

Nosema is an important disease that negatively affects bees' performance by disrupting the digestive system of bees. The disease, which is known to be widespread in almost every part of the world except Central Africa, is a highly dangerous fungal infection for honey bees, which occurs with infection of honey bees' ventricular epithelial cells by *Nosema apis* and *Nosema ceranae*. Nosema infection, one of the most common diseases in honey bees, is more dangerous due to the adverse effects of the factor itself and secondary infections. It is one of the most important causes of honey bee colony losses. This study aimed to investigate the effectiveness levels of essential etheric oils against nosema infection. 21 colonies obtained from Ordu Region, equalized physiologically and divided into 7 groups. Nettle, laurel, eucalyptus, thyme, garlic oils (after dilution of 0.48%), and apple cider vinegar were given to each colony 6 times at 3 days intervals of 3 mL/L to 1:1 ratio syrup (prepared for each separate group). Adult bee samples were taken from all colonies before each additional feeding, and nosema spore counts were performed.

Introduction

Nosematosis, the important bee diseases, is seen as one of the main causes of colony losses and reduces the adult bee population with the intensive detection of *Nosema ceranae* in recent years. The disease could be acute or chronic, and colony losses vary between 10-100% depending on environmental conditions. Indigestion, abdominal bloating, shortened lifespan, and inability to fly with open wings, change of queen and eggs, yellow-brown foul-smelling, dark-coloured stool are seen in sick bees. The decrease in the colony population and honey yield, and colony losses can be seen (Morse & Flottum. 1997).

Bees infected with *N. apis* survive 18-54 days in sudden colony extinction, while those infected with *N. ceranae* die within 8-10 days. Nosemosis has been identified by microscopic examination in 60% of the apiary in Turkey, 14% of colonies were found infected in these apiaries, and clinical symptoms were determined in 14% of infected colonies (Muz, 2008).

N. apis and *N. ceranae* cannot be distinguished by microscopic examinations used in routine research; separating these two factors by molecular genetic methods is possible (Uygur & Girişgin, 2008).

In most 1260 samples taken from 420 enterprises belonging to the Beekeepers of the Eastern Black Sea Region (infection rate found 89% in spring and 38% in autumn), nosema spores were encountered. It is important to consider that seriously; nosema spores were encountered in the autumn period. While *Nosema ceranae* was detected in all the positive samples collected in the spring and autumn periods, *Nosema apis* was not encountered (Yılmaz et al., 2018).

Essential oils, also known as volatile oils, are aromatic fatty compounds obtained from different parts

of plants (flowers, buds, seeds, leaves, branches, bark, wood, fruit, and roots). These natural products are widely used in various fields, including perfume, cosmetics, aromatherapy, phytotherapy, spices, nutrition, and agriculture. The biological activities of essential oils, including antibacterial, antiviral, antifungal, and anti-inflammatory effects, are well known. These oils can also be effective against organisms such as nematodes, helminths, and insects. Essential oils are known by different names, such as volatile oil, etheric oil, scented oil, or essence oil (Şengezer & Göngör, 2008).

Observing the antimicrobial effect, even in a microorganism, has shown that plants containing etheric oil can be used for therapeutic purposes and may be an alternative to synthetic antibiotics (Benli & Yiğit, 2005).

In recent years, the emergence of antibiotic-resistant microorganisms and being side effects not seen or seen less in natural medicines, which synthetic drugs have, led scientists to investigate natural-based treatments (Dürger, Ceyhan, Alitsaous, & Uğurlu, 1999).

Maistrello et al. (2008) evaluated the effects of some natural compounds (thymol, vetiver essential oil, lysozyme, and resveratrol) on *Nosema ceranae* spore infected honey bees by cage trials. Infected bees were given sugar solutions containing thymol 0.12 mg/g, lysozyme 2.5 mg/g, vetiver essential oil 1.2 mg/g, resveratrol 0.001 mg/g, and ethanol as a control group in the study. On the 8th day, 2.6 million spores/bees were detected in the control group, while on the 25th day, this number was determined as 230 million spores/ bees. Honey bees fed diets containing thymol, vetiver essential oil, lysozyme, and resveratrol; on the 8th day, 2.46 million spores/bees, 1.37 million spores/ bees, 1.87 million spores/bees, and 3.3 million spores/bees were identified, respectively. On the 25th day, 20.0 million spores/bees in the thymol group, 144.5 million spores/bees in the vetiver essential oil group, 54 million spores/bees in the resveratrol group were identified, and all bees of the lysosome group died. As a result, it was emphasized that the infection rates of bees fed with sugar solutions containing thymol and resveratrol decreased significantly and may be useful in alternative strategies for controlling nosema disease.

In the in-vitro study, in which Costa, Lodesani, and Maistrello (2009) evaluated the effects of thymol and resveratrol on the growth of *Nosema ceranae* and lifetime of honey bees, 100 ppm thymol and 10 ppm resveratrol were given to honey bees infected with of *Nosema ceranae* spore by adding 50% sucrose. The nosema spore numbers of honey bees fed with sugar solutions containing control, thymol, and resveratrol were counted as 2.0 million spores/bees, 1.7 million spores/bees, and 1.3 million spores/bees on the 8th day; 118.1 million spores/bees; 60.0 million spores/bees and 126.2 million spores/bees on the 25th day, respectively. On day 25 of the study, it is determined that bees fed with thymol syrup had significantly lower infection levels than control group bees, and bees fed thymol or resveratrol syrup were significantly live longer (23 and 25 days, respectively) than control group bees (20 days). Syrup, specially treated with thymol, has been reported to be promising in the control of nosema infection.

The activity of plant extracts on the development of *Nosema ceranae* was studied by Porrini et al. (2011). Honey bees were infected with nosema spores under laboratory conditions. Infected bees were fed ad libitum with enriched syrups. Diets in the feed of bees were created from ethanolic extracts from *Artemisia absinthium*, *Allium sativum*, *Laurus nobilis*, and *Ilex paraguariensis* diluted in syrup concentrations of 1% and 10%. On the 19th day after infection, it showed that the concentration of 1% *L. nobilis* extract significantly inhibited *N. ceranae*.

This study aimed to investigate the effectiveness levels of essential etheric oils against nosema infection.

Materials and Methods

21 colonies were obtained from Ordu Region in May 2019, and they were divided into 7 groups after equalization, one of which was a control group in 3 colonies. Application groups have been formed with nettle (*Urtica dioica*), laurel (*Cucurbita pepo*), eucalyptus (*Eucalyptus globulus*), thyme (*Thymus vulgaris*), garlic (*Allium sativum*) oils, and apple cider vinegar. All the essential oils were in a 20 mL bottle, purchased from the same brand produced in Turkey, and stored in appropriate conditions. Apple cider vinegar and essential oils were added to the syrup at a ratio of 1: 1, 3 mL/L, and given 1 L to each colony, 6 times with an interval of 3 days. The essential oil and syrup mixture were prepared daily. The oils belonging to the application groups were added to the syrup after 0.48% dilution. Only sugar syrup was given to the control group. Before each additional feeding, 10 worker bee samples were taken from each of the colonies, and nosema spore counts were performed.

Native Examination

By taking the abdominal intestine of 10 adult bees from each colony; Crushed in mortar in 2-3 ml saline. The native examination was performed under a light microscope with a 40X magnification after the suspension of the obtained bees obtained from adult bees on a lame with a lamella (Anonymous, 2005; Anonymous, 2009; Zeybek, 1991).

Staining Examination

After the samples prepared in physiological saline solution were air-dried and determined with ethanol, staining of spores was performed when stained with Giemsa for 45 minutes (Anonymous, 2009; Beyazıt et al., 2012).

Nosema Spores Counting

Samples were examined under a microscope, and spores counts (Shimanuki & Knox, 2000) were made from 20 bees of positive samples with nosema infection. At least 20 alive or dead bees suspicious of the disease were taken and kept in a container containing 70% alcohol for 1 day. The received bees were taken individually on a suitable ground (wood), and the abdomen was separated with the help of a scalpel. The cut abdomen was placed in a glass homogenizer or a suitable tube. A milliliter of water for each abdomen (20 mL of water for 20 abdomens) was added to the container. This mixture was thoroughly crushed with a rammer in a container. It was filtered with a filter paper to another container, filtered liquid mixed with a pipette and a drop placed to a lame, examined in a 40 lens after covering with lamella.

For the spores count, 5 large squares in the counting area of the 40 lenses were counted after the suspicious liquid was placed with a pipette to cover the Thoma slide area (a, b, c, d, e) (Figure 3). The total number of spores (S) applies to the formula: The number of spores per bee (N) = S × 4 × 106/80 by replacing the number in the formula, the number of spores per bee was calculated.

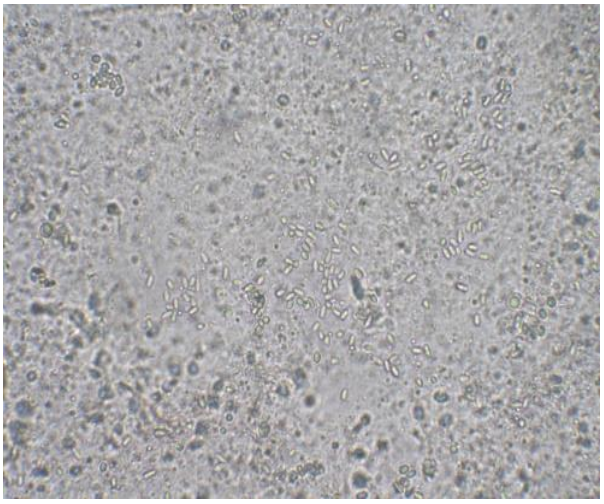

Figure 1. Native examination.

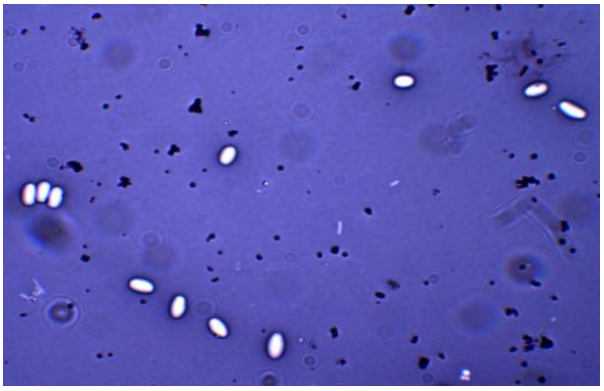

Figure 2. Colourization by examination.

Results

The intragroup normality assumption of the data was analyzed with the Shapiro Wilk test, and it was determined that the data were normally distributed ($P > 0.05$). Therefore, the data analysis was made according to the two-way analysis of variance in repeated measurements. Since Mauchly's sphericity test provides assumptions ($P = 0.36$), the results were examined according to the Sphericity assumption test.

The effect of the Essential oils and Essential oils x application time interactions were found insignificant. Since the difference between the application times was significant, comparisons were made according to Bonferroni test. SPSS package program was used to analyses of the data.

Boland, Fro, and Lorenz (1991) stated that the components found in plant oils such as eucalyptus, lemon extract, and thyme are effective against fungi.

It has been observed that the nosema spores in the application and control groups decreased significantly in the 3 weeks since the beginning of the study. The decreasing tendency was also decelerated in the 4th and 5th weeks (Table 1).

The importance of protective measures in beekeeping is revealed by the absence of a chemical drug against nosema, the desired result from alternative treatment methods, and the tendency of nosema infection in the control group to fall before the main nectar flow. By providing good ventilation of colonies against nosema infection, the formation of a moist environment that causes spores' proliferation can be prevented. Better results can be obtained with proper care and feeding activities. Colonies were observed during the essential oil trials, and no abnormal deaths were observed in any colonies.

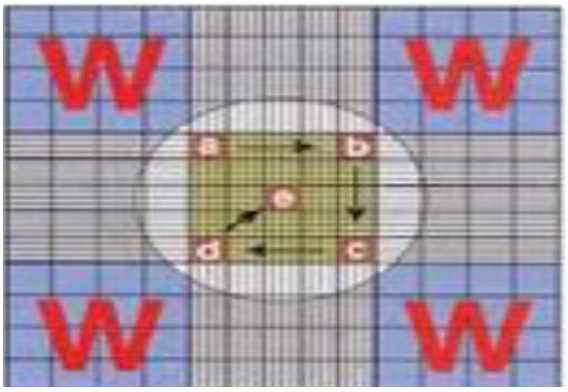

Figure 3. Nosema spores counting area

Discussion

As seen in Figure 4, spores' density in the control group and other groups decreased gradually after the four applications with an interval of 3 days, in the colonies emerging from the spring. In later measurements, the laurel and the control groups were constant. Simultaneously, an undesirable increase was observed in the thyme and eucalyptus, whereas there was a partial decrease in nettle, especially garlic.

Based on the findings here, it is thought that nosema spores can progress in the form of a spontaneous increase or decrease depending on the season and climate conditions.

Although the essential oils used in thyme and eucalyptus showed a better decrease in the first four applications than the control group, the tendency to increase again in the following applications suggests that this product may affect the intestinal tract flora of honey bees. Essential oils containing thyme and eucalyptus should be used in a maximum of 4 doses against Nosema infection and should not be exceeded.

Table 1. Spores intensity distribution during working time.

Essential oils	Weeks (Average ± SS)								
	n	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks	6 Weeks	n	Average ± SS
Nettle	3	34116666.7 ± 1233220.7	30233333.3 ± 7695182.5	12300000 ± 2961418.6	8066666.7± 2503164.7	9100000± 9860527.4	7633333.3± 5653612.4	18	16908333.3 ± 12295865.5
Laurel	3	28700000 ± 8886928.6	26816666.7 ± 7956339.2	17466666.7 ± 3859188.2	9516666.7 ± 1841421.6	8750000 ± 3001249.7	9100000 ± 8983874.4	18	16725000 ± 10172183.1
Thyme	3	19416666.7 ± 4365012.4	12516666.7 ± 6771693.5	8400000 ± 4411065.6	9033333.3 ± 3167149.1	6933333.3 ± 1409196	7866666.7 ± 1940575.5	18	10694444.4 ± 5578263.7
Ocaliptus	3	22250000 ± 14104343.3	19183333.3 ± 6824465.8	11450000 ± 476969.6	7950000 ± 4036087.2	8133333.3 ± 381881.3	9100000 ± 7668278.8	18	13011111.1 ± 8449600.7
Garlic	3	28966666.7 ± 12576797.4	31150000 ± 4130072.6	15450000 ± 3950949.3	11500000 ± 5358871.1	6000000 ± 1477328.7	3816666.7 ± 725143.7	18	16147222.2 ± 11990381.8
Vinegar	3	26716666.7 ± 1068098	26133333.3 ± 6130320.8	20983333.3 ± 5461761	16150000 ± 5438060.3	14900000 ± 8448520.6	11033333.3 ± 7821338.3	18	19319444.4 ± 7925427
Control	3	26866666.7 ± 10876158.9	26750000 ± 3553871.1	18683333.3±4 668065.3	14833333.3 ± 6984685.6	10033333.3 ± 5189010.8	10250000 ± 650000	18	17902777.8 ± 8828887.6

Variation sources	Plant	Time	Plant*Time
P	0.066	<0.001	0.384

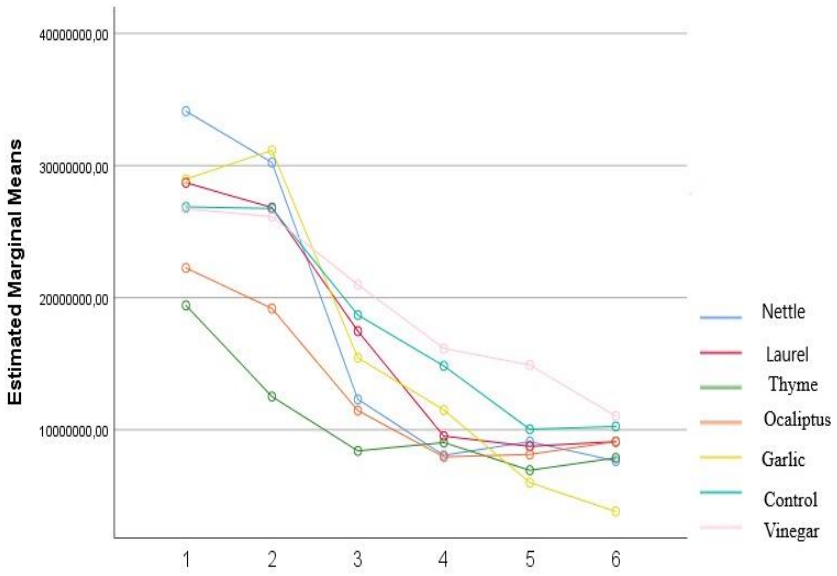

Figure 4. Spore intensity distribution after six applications of essential oils

Conclusion

Colony losses without reason have frequently been on the agenda in recent years, and the colonial losses must be associated with Nosemosis density in colonies. Protective measures should be taken against Nosema, and studies on treatment should be emphasized.

The study has shown that the essential oils used to decrease the number of spores that cause infection, but there is no definite result for treating the disease. It has been determined that garlic oil is one of the essential oils that has shown better results, and apple cider vinegar has no effect.

Essential oils used against nosema should be applied based on scientific data to protect human and bee health. The questions on the dose and mechanism of essential oils action against nosema infestation are not clear yet; more scientific studies are needed.

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RESEARCH PAPER

A Preliminary Study for the Application of ISSR Markers to Discriminate Honey Bee (*Apis mellifera* L.) Populations in Turkey

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Abstract

Potential of Inter Simple Sequence Repeats (ISSR) markers in analyzing and discriminating of Turkish honeybee populations was assessed in this study. Genomic DNA samples of workers belonging to four subspecies and two ecotypes of honey bees were amplified by PCR using 5 microsatellite primers. Principal component and population structure analyses showed that ISSR method successfully defined and discriminated four distinct Turkish honeybee subspecies while the two ecotypes of *Anatoliaca* subspecies were not distinctively separated from each other. This study showed that ISSR markers can be a simple and low-cost alternative with high resolution compared to other markers such as microsatellite and RAPD, in analyzing the genetic variations in Turkish honey bee subspecies.

Introduction

Anatolia's diverse climatic and geologic structure and its location as a bridge between Europe, Asia, and Africa led a considerable honey bee biodiversity in this region represented by five subspecies: *A. m. caucasica* at black sea region and northeastern Anatolia, *A. m. carnica* at Thrace region of north western Anatolia, *A. m. anatoliaca,* at western and central Anatolia, *A. m. meda* at southeastern Anatolia, and *A. m. syriaca* at Syria border region of southeastern Anatolia, (Ruttner, 1988; Kandemir, Kence, & Kence, 2000; 2005)

Honeybee diversity in Turkey was extensively studied using allozyme polymorphism, mitochondrial DNA, RAPD and microsatellite markers (Smith, Slaymaker, Palmer, & Kaftanoglu, 1997; Palmer, Smith, & Kaftanoglu, 2000; Bodur, Kence, & Kence, 2007; Tunca & Kence, 2011; Karabağ, Tunca, Sevim, & Doğaroğlu, 2020). Inter-SSR markers is based on amplification of genomic DNA segments flanked by the inversely oriented and closely spaced within microsatellite loci using microsatellite core unit bearing oligonucleotide primers. It combines the elements of microsatellite and RAPD methods (Wu, Jones, Danneberger, & Scolnik, 1994). ISSR has fewer time and material cost compared to microsatellite analysis as it does not require designing specific primers for the taxon to be analyzed (Zietkiewicz, Rafalski, & Labuda, 1994). It also produces more polymorphism compared to RAPD (Gupta, Chyi, Romerao-Severson, & Owen, 1994) ISSR was previously used to analyze the genetic differences in honeybee populations of Saudi Arabia (Al Otaibi, 2008) and Lithuania (Čeksteryte, Paplauskiene, Tamasauskiene, Pasakinskiene, & Mazeikiene, 2012). The aim of the study is to assess the utility of ISSR markers to discriminate some honeybee populations of different

subspecies and regions in Turkey.

Materials and Methods

Worker honeybees were sampled from hives of pure line native subspecies maintained in the common garden of METU Biology Department. Subspecies used in this study are: *A.m. caucasica* from Artvin province, *A.m. syriaca* from Hatay, *A.m. carnica* from Kırklareli and, Yığılca and Muğla ecotypes of *A.m. anatoliaca*. Head and thorax parts honeybee samples which were frozen at -80ºC were dissected out and homogenized in ice cold TE buffer. DNA extraction from the homogenates were performed using Fermentas DNA isolation kit according to manufacturer's instructions. DNA concentration and quality in samples were determined by Nanodrop 2000c spectrometer. Five of SSR primers belonging to a primer set prepared by Biotechnology Laboratory of University of British Columbia (Vancouver, Canada) were used in PCR amplification (Table 1). ISSR-PCRs were carried out in a Techne TC-5000 gradient thermal cycler and reaction mixtures were prepared in a total volume of 15 µL each containing 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 2 mM of $MgCl_2$, 200 µM of each of the dNTPs (dATP, dCTP, dGTP and dTTP), 0.25 µM of primer,0.5UT a qDNA polymerase (MBI, Fermentas) and 25 ng of template DNA. Time and thermal profile of the PCR were as follows 2 min of initial denaturation at 94°C, followed by 34 cycles; each consisting of a denaturation segment of 20 s at 94°C, an annealing segment of 1 min at 54 to 56°C depending on the primer and an extension segment of 2 min at 72°C. PCR was terminated with a final extension period of 5 min at 72°C, and products were electrophoresed in 1.5% agarose gels along with one lane of size markers. Fragments were visualized on a UV transilluminator following staining with 0.4 µg/mL ethidium bromide solution (Figure 1).

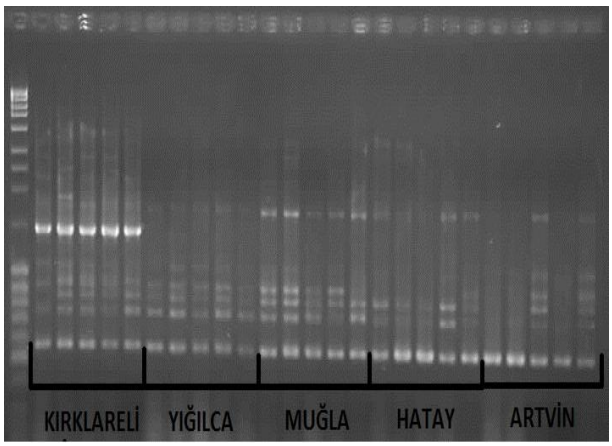

Figure 1. An example of PCR profiles of honey bees belonging to five populations.

Screening 150 honeybee samples with 5 SSR primers produced at 19 gel positions (loci). Each locus was treated as a separate character, and the presence or the absence of a PCR product at each position was scored as 1 and 0 respectively. Principal Component analysis (PCA) was performed by NTSYS-pc program to define and discriminate study samples. Population structure and possible hybridizations between samples were analyzed by Structure Software. Other genetic variation parameters were calculated by PopGene Program

Results

A total of 150 bee samples from 5 populations were screened with 5 primers in this study. In terms of genetic variation parameters (Table 1) populations showed a gradual increase with Artvin (*caucasica)* has the lowest while Kırklareli *(carnica)* had the highest scores (Table 2). The two- and three-dimensional graphics (Figures 2 and 3) of PCA analysis shows that the sample groups of four subspecies involved in our study, clearly separate from each other while Yığılca and Muğla ecotypes of *anatoliaca* subspecies are generally grouped together. Population structure analysis (Figure 4) also show distinctive patterns for each subspecies although there is some degree of introgression between the populations. Yığılca and Muğla ecotypes of *anatoliaca* honeybee also show remarkably similar structural patterns.

Discussion and Conclusion

Studies showed evidence of genetic homogenization and loss of genetic diversity in some areas of Europe. Dall'Olio, Marino, Lodesani, and Moritz (2007) reported high level of homogenization and decrease in previously reported genetic diversity in Italian peninsula. Canovas, de la Rua, Serrano, and Galian (2011) also found an increased level of homogenization in Iberian honeybee (*A. m. iberiensis*) colonies from Spain. Bouga et al. (2005 a,b) observed no genetic distinction in terms of mitochondrial and allozyme data between native *adami, macedonica* and *cecropia* subspecies in Greece. The main reasons for this phenomenon are migratory beekeeping and replacing the queens of native honeybee colonies with commercial breeds of other subspecies (especially *caucasica* in Turkey) and their hybrids (Kükrer, 2013). Therefore, a simple, fast and cost-effective method is needed for genetic screening for native honeybee subspecies to detect possible homogenization and introgression events.

ISSR markers appeared to be an effective tool in analyzing and discriminating different honeybee populations and subspecies. Population structure analysis show distinct patterns between subspecies with some degree of hybridization. This is consistent with

Table 1. Primer sequences and numbers of gel positions scored.

Primers	Sequences 5'→3'	Total Bands	Polymorphic bands	Percentage of polymorphic Bands
UBC 818	CAC ACA CAC ACA CAC AG	3	2	66
UBC 840	CAC ACA CAC ACA CAC AG	5	4	80
UBC 847	CAC ACA CAC ACA CAC ARC	4	4	100
UBC 856	ACA CAC ACA CAC ACA CYA	5	4	80
UBC 899	CAT GGT GTT GGT CAT TGT TCC	2	2	100

Table 2. Genetic variation and polymorphism in different indigenous honeybee populations from Turkey. NPL number of polymorphic loci, PPL percentage of polymorphic loci, *Na* observed number of alleles per locus, *Ne* effective number of alleles, *H* Nei's gene diversity *I* Shannon's Information index.

Populations	NPL	PPL	NA	NE	H	I
Artvİn	10	50	1.500±0.513	1.250±0.361	0.146±0.196	0.223±0.280
Hatay	11	55	1.550±0.510	1.268±0.358	0.160±0.193	0.246±0.275
Kırklarelİ	12	60	1.600±0.502	1.393±0.389	0.228±0.207	0.337±0.297
Muğla	14	70	1.700±0.470	1.422±0.372	0.248±0.200	0.370±0.283
Yığılca	17	85	1.850±0.366	1.425±0.351	0.257±0.178	0.395±0.241

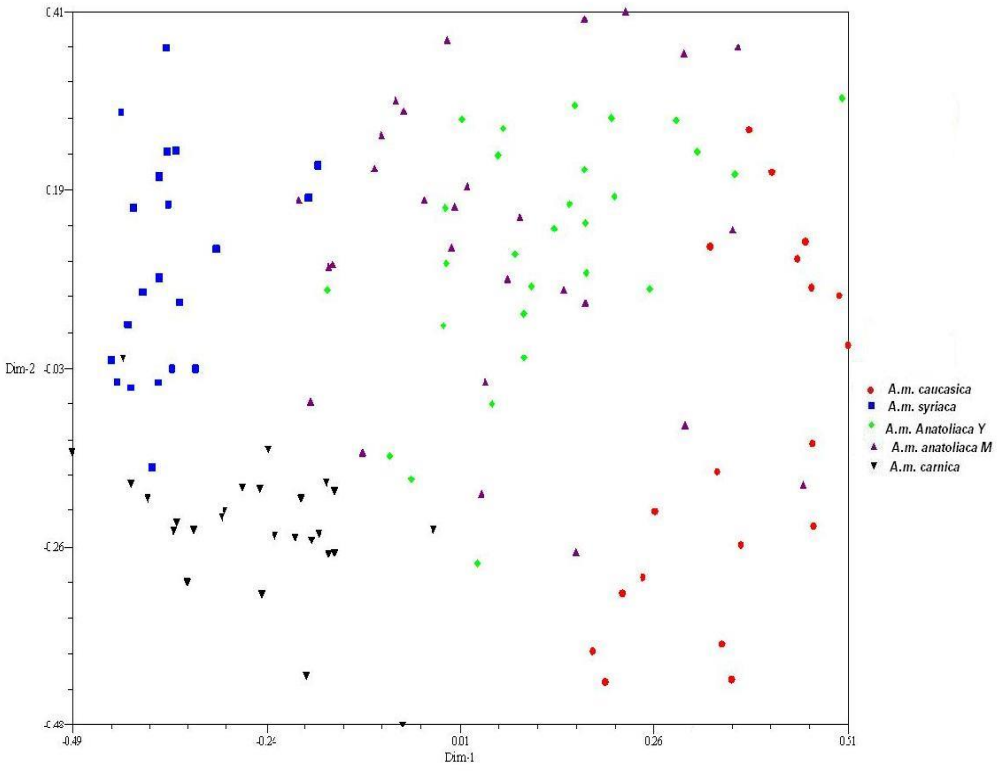

Figure 2. Two-dimensional principle coordinate analysis of 150 honey bees from 5 populations according to a distance matrix based on Jacard Index.

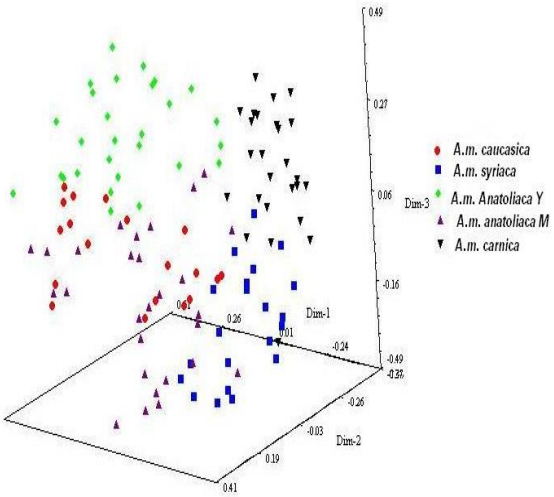

Figure 3: Three- dimensional principle coordinate analysis of 150 honey bees from 5 populations according to a distance matrix based on Jacard Index.

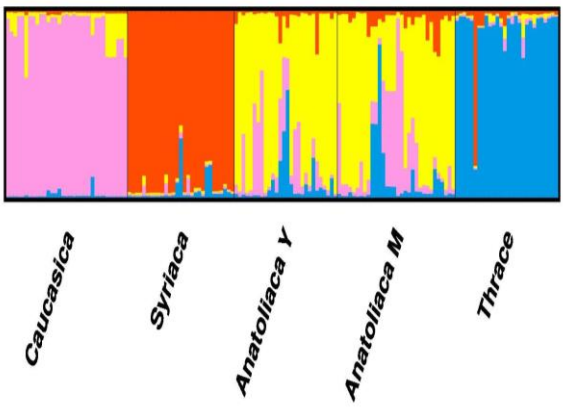

Figure 4: Structure analysis for five honey bee populations of Turkey.

previous studies which showed that honeybee populations in different regions of Anatolia still retain their distinctive genetic structures despite hybridization caused by migratory beekeeping (Tunca, 2009, Kükrer, 2013). In addition to this, Yığılca and Muğla ecotypes of *A. m. Anatoliaca* subspecies are revealed to be genetically quite similar even though they are adapted to different ecological conditions. In conclusion, ISSR method proved itself to be a simple and low-cost method capable of identifying and discriminating honeybee subspecies and populations of Turkey.

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RESEARCH PAPER

Antibiofilm, Antioxidant and Quorum Quenching Activities of Propolis Samples from Southwest Anatolia

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Abstract

In this study, antibiofilm, antioxidant and quorum quenching activities of the ethanol extracts of propolis samples collected from Muğla district were investigated. Antimicrobial activity was determined using the well diffusion and broth tube dilution methods, antibiofilm activity with microplate biofilm method, and antioxidant activity with DPPH radical scavenging, β-carotene linoleic acid and ferric thiocyanate methods. To determine the antimicrobial activity of the extracts, *Listeria monocytogenes* ATCC 7944, *Streptococcus mutans* CNCTC 8/77, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028 and *Candida albicans* ATCC 10239 strains were used. Minimum inhibitory concentrations (MIC) against microorganisms were determined from 1 to >100 mg/ml. The lowest MIC value was found as 1 mg/ml for AP6 propolis extract against *Salmonella typhimurium.* According to the antibiofilm activity results, highest biofilm were detected at concentrations of MIC as 82.60% for AP1 against *S. mutans*, 67.45% for AP2 against *L. monocytogenes*, 73.02% for AP3 against *S. mutans*, 64.05% for AP4 against *L. monocytogenes*, 70.58% for AP5 against *S. typhimurium*; 93.43% for AP6 against *S. typhimurium* and 72.43% for AP7 against *S. mutans*. AP7 extract had the highest antioxidant activity with an IC_{50} value of 3.94 mg/ml for DPPH radical scavenging method and with 91.10% reduction rate for β-carotene linoleic acid method. AP1 extract had the highest reduction percentage rate of 51.77% in the ferric thiocyanate method.

Introduction

Propolis is a natural resin, collected mainly by the honey bee, *Apis mellifera*, which has been shown to have many biological activities including antioxidant and antimicrobial effects, both conferred by phenolic compounds, especially flavonoids (Gonçalves, Santos, & Srebernich, 2011; Talla et al., 2017; Tamfu et al., 2020). More than 150 components such as polyphenols, phenolic aldehydes, sesquiterpene quinines, coumarins, amino acids, steroids and inorganic components have been identified in propolis samples (Marcucci, 1995; Anjum et al., 2019). Propolis has long been used in oriental folk medicine for curing infections (Cheng & Wong, 1996; Blicharska & Seidel, 2019) and in European ethno-pharmacology as an antiseptic and anti-inflammatory agent for healing wounds and burns (Ghisalberti, 1979; Rojczyk, Klama-Baryla, Labus, Wilemska-Kucharzewska, & Kucharzewska, 2020). Propolis exhibits antimicrobial, antioxidant, anti-inflammatory, anaesthetic and other properties (Bankova, de Castro, & Marcucci, 2000).

The purposes of the study were to determine antibiofilm, antioxidant and quorum quenching activities of propolis samples.

Materials and Methods

Propolis samples and preparation of alcohol extracts

Propolis samples were collected from seven different areas; Marmaris (Osmaniye: AP1; Merkez: AP5); Fethiye (Yanıklar: AP2; Uzunyurt: AP3); Datça (AP4); Milas (AP6); and Bodrum (Gümüşlük: AP7) in Muğla located in Southwest Anatolia. Each sample was cut into small pieces after cooling at -20°C and extracted with 96%

ethanol (1:10 w/v) at 37°C for 5 days. The ethyl alcohol extracts were then filtered through a Whatman No. 1 filter paper and evaporated to dryness under vacuum. The samples were kept at -20°C until test experiments (Blonska et al., 2004).

Antibiofilm Activity

Antibiofilm activities in MIC, MIC/2, MIC/4 and MIC/8 concentrations for propolis extracts were determined on polystyrene flat-bottomed microtitre plates as described by Merritt, Kadouri and O'Toole, (2005).

Antioxidant Methods

Determination of DPPH Radical Scavenging Activity

Antioxidant activity of the propolis extracts were determined based on its ability to react with the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Burits & Bucar, 2000) Percentage of inhibition and the concentration of sample required for 50% scavenging of the DPPH free radical (IC_{50}) were determined. BHT and ascorbic acid were used as a positive control.

β-carotene Linoleic Acid Methods

β-Carotene-linoleic acid test system was used to assay lipid-peroxidation inhibitory activity (Dapkevicius, Venskutonis, van Beek, & Linssen, 1998).

Ferric Thiocyanate (FTC) Method

A screw-cap vial containing a mixture of 4 mg of sample in 4 mL of 99.5% ethanol, 4.1 mL of 2.51% linoleic acid in 99.5% ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0) and 3.9 mL of water (final concentration 0.02%, w/v) was placed in an oven at 40°C in the dark (Mitsuda, Yuasumoto, & Iwami, 1996). To 0.1 mL of this mixture in a test tube, 9.7 mL of 75% (v/v) ethanol, 0.1 mL 30% ammonium thiocyanate and finally, 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture. Three minutes after the addition of ferrous chloride, the absorbance was measured at 500 nm. This step was repeated every 24 h until the control reached its maximum absorbance value.

Quorum Quenching Activities Methods

The bacterial strains employed in the study were *Chromobacterium violaceum* CV026 for anti-quorum sensing, *C. violaceum* CV12472 for violacein inhibition effects of the propolis extracts. Anti-quorum sensing experiments were carried out according to the methods of Koh and Tam (2011), while the violacein inhibition experiments were performed as described by Choo, Rukayadi, & Hwang, (2006).

Results

Prior to investigation of biofilm inhibitory potential of test samples, MIC values of propolis samples were determined on the selected microorganisms and biofilm inhibition assay was performed at MIC and sub-MIC concentrations. The anti-biofilm activity results are given in Table 1 as percentage inhibition values. The antibiofilm activity results showed that the highest biofilm inhibition were observed at MIC concentrations. The antioxidant potential of propolis samples were evaluated using three different methods: DPPH radical scavenging assay, β-carotene-linoleic acid assay and Ferric thiocynate method and the results presented on Figure 2-4. Prior to quorum quenching activity determination, the MIC values of the propolis extracts were determined against *C. violaceum* CV 12472 and CV026 and presented in Table 2. The MIC and sub-MIC concentrations were then used for the determination of percentage violacein inhibition of samples (Table 3, Figure 1). The MIC values of the propolis extracts against *C. violaceum* CV026 biomonitor strain were determined and their QSI evaluated at sub-MIC concentrations (Table 4).

Discussion

Antibiofilm Activity

The biofilm inhibitions were determined as 82.60% for AP1 sample against *S. mutans*, 67.45% for AP2 against *L. monocytogenes*, 93.43% for AP6 against *S. typhimurium* for MIC concentration. Scazzocchio, D'Auria, Alessandrini, and Pantanella (2006) found a higher rate of *S.aureus* biofilm inhibition than our study. Koudhi, Zmantar, and Bakhrouf (2010), Dogan et al. (2014) and Capoci et al. (2015) reported higher rates of biofilm inhibition at lower concentrations compared to our results. This can be caused by the difference in the regions of collection of propolis samples.

The Antioxidant Activities

DPPH radical scavenging, β-carotene linoleic acid and ferric thiocyanate methods were used for determination of antioxidant activities. The extracts of the propolis sample from Bodrum, Gümüşlük (AP7) showed the highest antioxidant activities with IC_{50} value of 3.94 mg/mL for DPPH radical scavenging method and with 91.10% reduction rate for β-carotene linoleic acid method. The lowest antioxidant activity was determined as IC_{50} 26.33 mg/mL for DPPH radical scavenging method in AP2. The highest antioxidant activity was shown at AP1 extract with a rate of 51.77% using ferric thiocyanate method whereas AP4 extract showed the lowest prevention of lipid peroxidation (34.74%). Nieva Moreno, Isla, Sampieto, and Vattuone (2000) and Lu, Chen, and Chou (2003) reported results consistent with

Table 1. Antibiofilm activities of propolis extracts at the MIC and sub-MIC concentrations

Extracts	Conc. (mg/mL)	*C. albicans*	*S. aureus*	*S. mutans*	*L. monocytogenes*	*E. coli*	*S. typhimurium*
AP1	MIC	10.99±5.84	47.28±4.19	82.60±6.92	60.63±4.75	3.29±2.36	42.99±0.40
	MIC/2	-	29.55±3.53	73.11±0.14	48.49±5.35	-	35.50±0.60
	MIC/4	-	11.70±5.20	71.85±0.23	32.96±4.53	-	28.03±0.26
	MIC/8	-	-	62.96±0.15	18.42±1.76	-	5.59±0.88
AP2	MIC	-	35.37±2.85	46.21±7.84	67.45±1.77	8.88±0.55	60.23±4.57
	MIC/2	-	10.39±0.63	36.45±2.73	53.89±1.00	-	40.64±1.02
	MIC/4	-	1.76±3.38	21.95±1.02	45.62±0.52	-	26.63±0.21
	MIC/8	-	-	-	27.62±5.07	-	7.48±1.00
AP3	MIC	14.59±1.19	65.24±0.20	73.02±1.39	18.96±3.58	10.28±0.09	38.78±0.10
	MIC/2	5.16±4.11	53.62±0.78	56.77±1.36	-	-	28.01±2.54
	MIC/4	-	39.53±4.57	31.04±2.67	-	-	10.73±1.30
	MIC/8	-	27.19±4.42	14.03±4.57	-	-	-
AP4	MIC	10.92±1.44	32.76±1.05	61.98±1.52	64.05±2.29	9.34±0.08	58.41±0.07
	MIC/2	7.79±1.48	23.88±1.11	50.58±0.58	51.45±0.47	-	41.07±5.22
	MIC/4	6.74±2.53	7.37±0.75	32.21±3.83	38.77±6.41	-	22.88±1.18
	MIC/8	1.56±0.53	-	8.51±3.11	24.21±5.59	-	3.72±1.83
AP5	MIC	51.53±3.10	46.58±2.68	61.40±2.10	70.41±3.11	13.56±1.52	70.58±2.06
	MIC/2	26.58±1.83	31.29±0.41	40.90±3.69	62.67±5.94	-	44.88±3.22
	MIC/4	-	15.40±0.77	35.02±2.99	50.92±4.84	-	19.57±5.42
	MIC/8	-	-	10.63±0.17	34.94±0.63	-	1.88±1.88
AP6	MIC	28.14±3.37	26.41±4.46	86.47±5.38	68.43±0.79	14.50±2.47	93.43±1.93
	MIC/2	13.58±4.30	18.50±0.61	61.98±1.52	36.37±3.04	-	82.73±2.17
	MIC/4	7.29±0.07	-	32.40±1.31	12.66±4.00	-	75.22±0.69
	MIC/8	-	-	18.85±1.41	-	-	49.07±0.92
AP7	MIC	59.88±0.93	54.15±1.94	72.43±0.81	61.12±4.26	12.10±4.55	64.45±3.13
	MIC/2	41.67±0.43	44.01±0.10	56.00±3.29	46.10±1.00	-	53.24±3.24
	MIC/4	24.97±2.86	26.28±0.54	39.26±1.42	32.98±2.59	-	35.97±1.06
	MIC/8	-	12.30±0.92	21.57±4.00	15.56±3.06	-	26.15±1.62

-: No activity

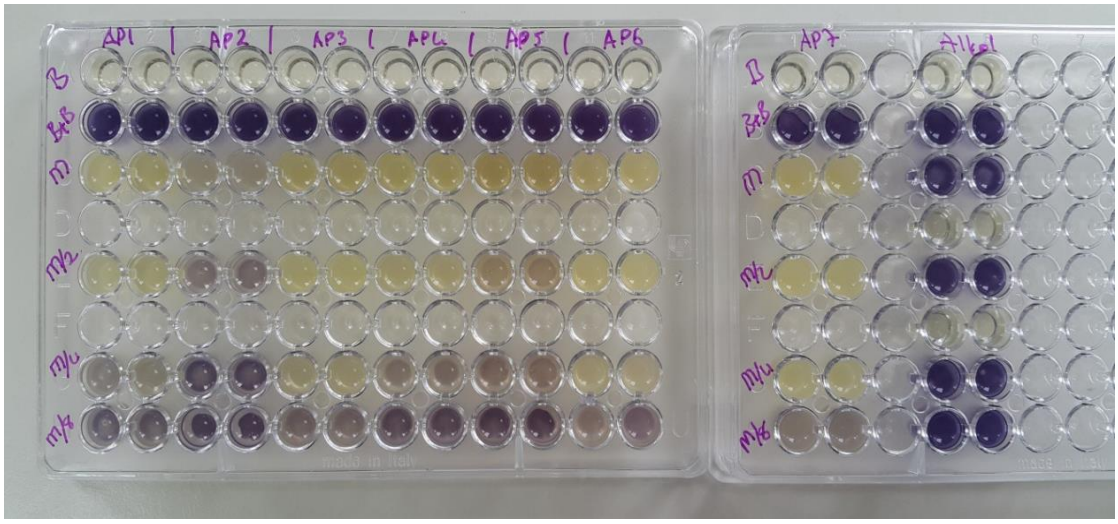

Figure 1. Violacein production inhibition results of propolis extracts against CV 12472

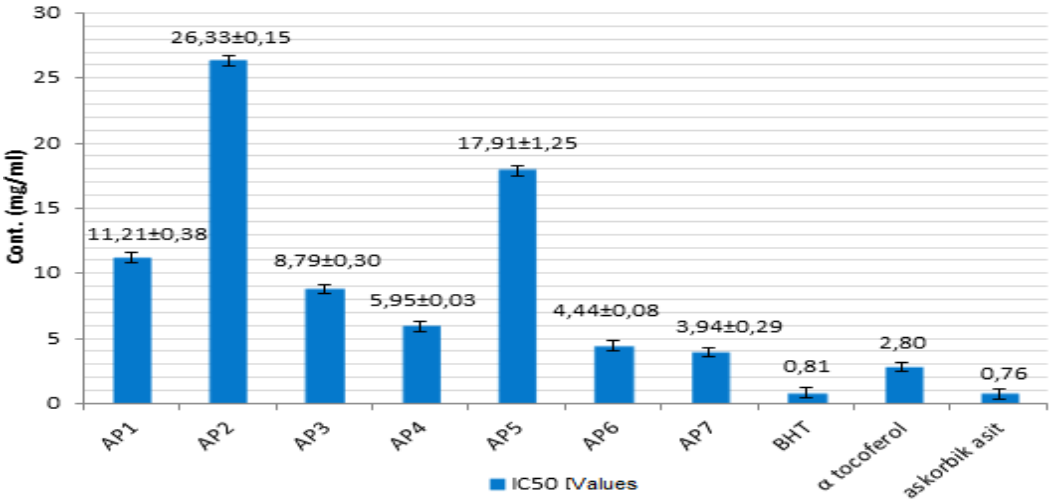

Figure 2. Results of DPPH free radical scavenging activities of propolis extracts,

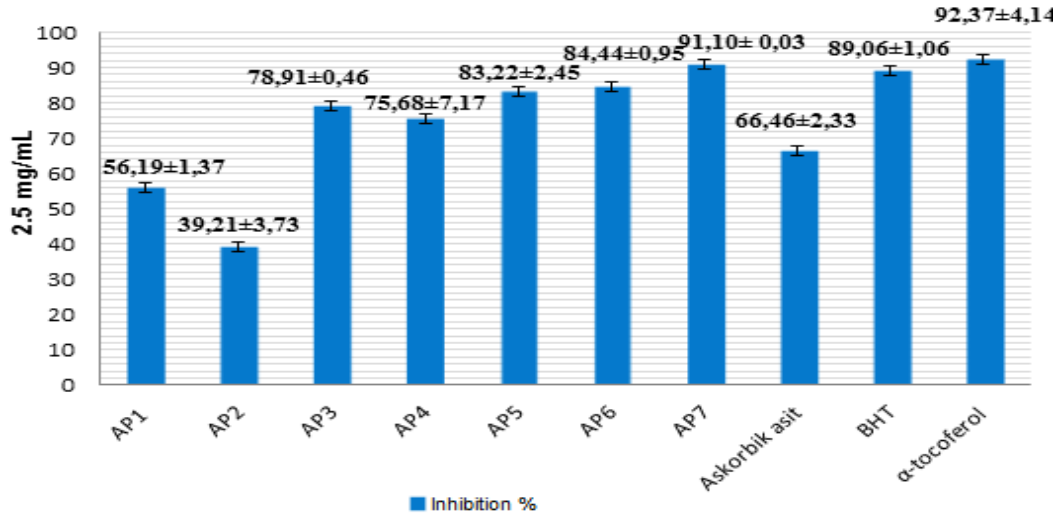

Figure 3. β-carotene-linoleic acid assay results of Propolis extracts

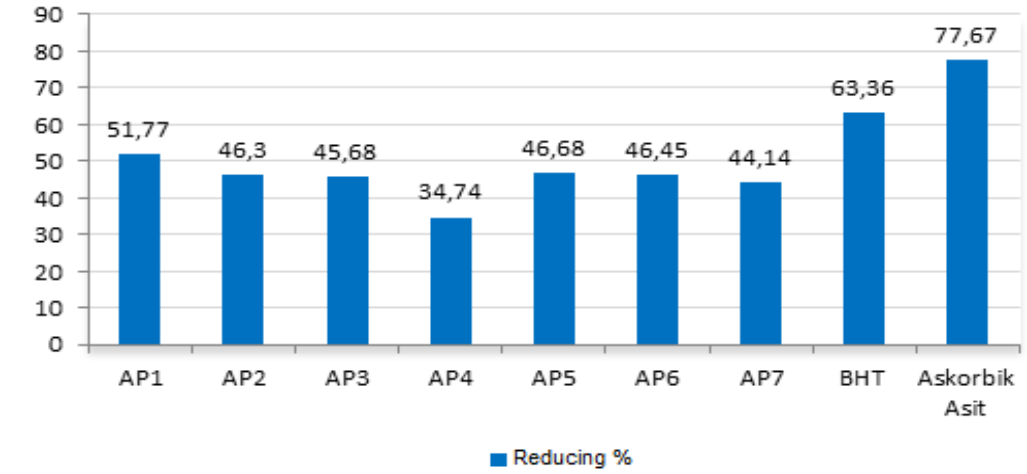

Figure 4. FTC result graph of propolis extracts (% inhibition)

Table 2. MIC concentrations against *C. violaceum* CV 12472 and *C. violaceum* CV 026 strains

Samples	CV12472	CV026
	MIC Conc.(mg/mL)	
AP1	6.25	6.25
AP2	6.25	6.25
AP3	6.25	6.25
AP4	3.12	6.25
AP5	12.5	12.5
AP6	3.12	3.12
AP7	3.12	3.12

Table 3. Violacein inhibition rates of propolis extracts against *C. violaceum* CV 12472

	Violacein inhibition (%)						
Conc.	AP1	AP2	AP3	AP4	AP5	AP6	AP7
MIC	100	64.8±0.5	100	100	100	100	100
MIC/2	100	39.8±0.2	100	100	51.8±1.8	100	100
MIC/4	47.1±1.2	29.2±0.5	100	50.4±3.7	48.9±0.6	100	100
MIC/8	41.2±0.8	23.1±0.3	49.7±1.0	34.6±1.1	37.3±0.5	45.6±2.8	51.0±0.4

Table 4. Antiquorum sensing activity results of propolis extracts

Propolis extracts	Concentrations (mg/mL)	*C. violaceum* CV026	
		Antimicrobial zone (mm)	QS inhibition zone (mm)
AP1	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP2	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP3	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP4	MIC	-	9
	MIC/2	-	-
	MIC/4	-	-
AP5	MIC	-	-
	MIC/2	-	-
	MIC/4	-	-
AP6	MIC	8	10
	MIC/2	7	-
	MIC/4	-	-
AP7	MIC	-	12
	MIC/2	-	10
	MIC/4	-	8
C_{10}HSL		-	31
Ethanol		-	-

-: No effect

our DPPH results in their study. Chen, Weng, Wu, and Lin (2004), Russo et al. (2004), Choi et al. (2006), Alencar et al. (2007), Moreira, Dias, Pereira, and Estevinho (2008), Miquel, Nunes, Dandlen, Cavaco, and Antunes (2010), Piccinelli et al. (2013) and Silva Frozza et al. (2013) reported lower IC_{50} values compared to our results. Sheng, Zhou, Wang, Xu, and Hu (2007) reported results consistent with our study in the antioxidant activity experiments they conducted with the FTC method.

Quorum Quenching Activities

In the study, MIC values of biomonitor strains against propolis extracts were determined prior to the determination of antiquorum sensing activity. The highest antimicrobial effect was found in AP4, AP6 and AP7 extracts against C. violaceum CV12472 strain. The highest antimicrobial effect was seen in AP7 extract against C. violaceum CV026 strain. All of the propolis extracts used in the study showed inhibition of violacein production at different concentrations. Also, the highest inhibition of violacein production was determined in 100% at concentrations of MIC, MIC/2 and MIC/4, and 51.0% at a concentration of MIC/8 for AP7 extract.

It was determined that AP4, AP6 and AP7 extracts among the propolis extracts used in the study have antiquorum sensing activity. However, there were no activity detected for AP1, AP2, AP3, AP5 for antiquorum quenching activity. The results reported by Savka et al. (2015) confirm our anti-quorum sensing results.

Conclusion

The antimicrobial effect of propolis samples collected from Milas and Bodrum (Gümüşlük) was revealed in this study. It was found that the propolis sample collected from the Milas region highly inhibited the *S.mutans* and *S.typhimurium* biofilm formation, especially at MIC concentrations. DPPH and β-carotene linoleic acid method results also revealed that Milas and Bodrum (Gümüşlük) propolis samples have high antioxidant activity as well as antimicrobial activity. Considering the antiquorum sensing results, it was determined that the Bodrum (Gümüşlük) propolis sample was effective in terms of quorum sensing inhibition.

Amongst the propolis ethanol extracts used in the study, Milas and Bodrum (Gümüşlük) samples showed the feature of being alternative in the treatment of many infections due to their antimicrobial and antibiofilm activities, and to eliminate the harmful effects of free radicals that cause many health problems, according to the antioxidant activity results. In addition, these two propolis extracts were found to be able to prevent the quorum sensing communication system used by most pathogenic bacteria to cause disease and especially to control biofilm production. Accordingly, it has been revealed that these two extracts have a potential in alternative treatment studies to be applied in the field of medicine.

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