Original Article

FACTORS AFFECTING QUALITY OF HONEY BEE VENOM

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Abstract

Honeybee venom (HBV) is an important product of beehives, and its benefits for health have been rediscovered by modern medicine. Since HBV has the potential to treat some diseases, its quality and production conditions require a detailed investigation. The objective of this study is to understand how season, harvesting time (day or night), harvesting site of beehives (inside or entrance) and geographic location affects quality through the analysis of apamin, melittin and phospholipase A_2 (PLA2) content. Each set of six colonies were used to understand the differences in these components when HBV is harvested in the daytime either from the entrance or inside of the hives and nighttime from the inside of the hives. The experiment also investigated seasonal differences as the samples were harvested each month from May to August 2019 in our apiary. Furthermore, the effect of geographic location on quality was examined through the comparison of the data obtained from twenty-seven samples collected by beekeepers using the same device, located in the Turkish cities of Manisa, Muğla, Balıkesir, Düzce and Mersin. The results demonstrated that statistically significant differences in the amounts of analyzed components were not dependent on harvesting time, collection site on the beehives or season. On the other hand, region samples significantly differed in the amounts of all three components, ranging from 1.28% to 3.81% for apamin, 19.51-64.03% for melittin and 7.22%-28.18% for PLA2. However, beekeepers' improper practices during harvesting and storing might be the most critical parameters that determine the quality of HBV.

Keywords: apamin, harvesting method, honeybee venom, melittin, Phospholipase A2, season

INTRODUCTION

Honeybee venom (HBV) with various health benefits is one of the most significant new-generation hive products. Although it has been used in traditional medicine for centuries (Bogdanov, 2012; Cells, 2013; Hwang et al., 2015; Abdela & Jilo, 2016; Eze et al., 2016; Baher & Abo Zeid, 2017; Kader, et al., 2019; Kim et al., 2019; Kurek-Górecka et al., 2020), it has been recently rediscovered by modern medicine for its pharmacological value.

The therapeutic application of HBV in traditional and complementary medicine has been demonstrated to treat such autoimmune and inflammatory diseases as rheumatoid arthritis, osteoarthritis and chronic pain (Abdela & Jilo, 2016). It has also been used for the treatment of such neurological diseases as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Parkinson and Alzheimer's (Hwang et al., 2015). Additionally, antimutagenic, radioprotective, an-

tinociceptive, anti-inflammatory and anticancer effects of HBV have been described by many other studies (Varanda & Tavares, 1998; Kim et al., 2003; Lee et al., 2004; Son et al., 2007; Gajski & Garaj-Vrhovac, 2009; Mahmoodzadeh et al., 2013; Kolayli & Keskin, 2020; Uzuner et al., 2021). The pharmaceutical value of HBV has been referred to for its valuable ingredients, including proteins (phospholipase A_2 (PLA2), phospholipase B, hyaluronidase, phosphatase, alpha glucosidase), peptides (apamin, melittin, MCD peptide, secapine, pamine, minimine, adolapine, procamine A and B, protease inhibitor, tertiapine, cardiopep, melittin F), phospholipids, biogenic amines (histamine, dopamine, noradrenalin), amino acids (aminobutyric acid, alpha-amino acids) in addition to sugars, minerals (P, Ca, Mg), and volatile compounds (pheromones) (Bogdanov, 2012).

Consequently, the production and commercialization of HBV are being developed in Turkey and the world due to a growing interest in its potential as an ingredient for cosmetics and health products. Thus, its production and further processing conditions have become decisive parameters among the producers and the purchasers. According to previous studies, HBV quality is affected by the production method and environmental factors such as humidity, heat, and harvest time (Ghany Zidan, et al., 2018; Hussein et al., 2019). Also, some studies reported the influence of the age of honeybees (Owen et al., 1990; Owen, 1978; Bachmayer et al., 1972; Owen et al., 1974), season (Owen & Bridges, 1982; Owen & Sloley, 1988), and supplementary feeding (Abusabbah et al., 2016; Badaway et al., 2016; Nowar, 2016) on its constituents. Currently, limited researchers, carried out in commercial samples of Anatolian HBV, provide information on its composition (Samancı & Kekeçoğlu, 2019; Kekeçoğlu et al., 2019, Şirin et al., 2016). However, these studies need to be enhanced through focusing on the factors determining its quality, specifically for Anatolian honeybee (*Apis mellifera anatoliaca*) venom.

High-quality HBV production is essential for the beekeeper, purchaser, and, most importantly, patients who is applied bee venom is concerned. Since HBV is a delicate product, even a little error during its production can affect its quality. Thus, good production techniques and the harvesting season of this unique food became a significant subject for the scientists.

This study aims to investigate how harvesting time, beehive's site, season and geographic location affect the three main components of Anatolian HBV, apamin, melittin, and PLA2. We expect the study results to stimulate future research focusing on a standard quality of bee venom by guiding good production principles associated with its components.

MATERIAL AND METHODS

HBV harvesting device

All samples, including the samples obtained from the beekeepers, were collected with the same brand electroshock-based HBV harvesting device with 1.6 mm stainless chrome wires and 5-second repeat electroshock waves with 2.2 watts.

HBV samples and harvesting techniques

The HBV samples were produced at the Düzce University Beekeeping Research Development and Application Centre (DAGEM)'s apiary under controlled conditions. The samples were obtained by the active operation of the electroshock-based device (Almışlar Arı Zehri Toplama Makinesi, product no: 1568) for fifteen minutes according to the methods of Bogdanov (2012) and Haggag et al. (2015) with slight modifications. Collected samples were transferred to the laboratory in a cold chain and kept at -18°C in the dark until the analysis.

Each set of six colonies, all equal strength, was used to study the factors of harvest time, the beehive's harvest site and harvest season on HBV quality. The daytime samples (DC-day time Collection) were collected either from the entrance (EH-entrance of the hive) or inside (IH-inside hive, from the top of the honey super) the hives but the nighttime samples (NC-Night-time Collection) from the hives' inside only. Day-time samples were collected between 01:00 PM and 04.00 PM, whereas night-time sampling

Table 1.

Sample groups described according to the selected factors [a]

Code for Sample Group[a]	Total Sample Numbers	Factors			
		Harvesting Time	Harvesting Site of the Beehive	Seasons	Geographic Location
IH / DC	16	Day	Inside	May, June, July, August	DAGEM Apiary, Duzce
EH/DC	17	Day	Entrance	May, June, July, August	DAGEM Apiary, Duzce
IH/NC	16	Night	Inside	May, June, July, August	DAGEM Apiary, Duzce
DS	50	Day time	Inside	May, June, July, August	DAGEM Apiary, Duzce
DGL	27	Day	Inside	-	Apiaries located in Manisa, Muğla, Balikesir, Düzce and Mersin

[a] IH/DC= Harvested from the inside of hive at day-time, EH/DC=Harvested at the entrance site of hive at day-time, IH/NC= Harvested from the inside of hive at night-time, DS=Difference in Seasons, DGL=Difference in Geographic Location

was carried out between 09.00 PM and 12.00 PM on the same day in a cycle of every fifteen days. At the beginning of the trial, we aimed to collect twenty-four samples for each group (IH/NC, EH/DC, IH/DC), but we were only able to proceed with seventeen IH/NC, seventeen EH/DC and sixteen IH/DC samples due to insufficient sample amounts for HPLC analysis. In addition, every month, from May to August, samples (DS: Differences in Seasons) were produced to evaluate the seasonal influence. Those samples were harvested during the daytime from the inside of the beehives using the above methods. Finally, the effect of geographic location on HBV quality was examined through the comparison of data for twenty-seven samples obtained by (DGL: Differences in Geographic Locations) beekeepers using the same device located in other cities (Manisa, Muğla, Balikesir, Düzce and Mersin) in Turkey.

The samples are presented in groups based on the selected factors for proper comparisons and sound interpretation (Tab. 1).

Chemicals

Chemicals used in the study are melittin from honeybee venom (Sigma-Aldrich, CAS=20449-79-0), apamin (Sigma-Aldrich, CAS=24345-16-2), phospholipase A2 from honeybee venom (Sigma-Aldrich, CAS=9001-84-7), purchased from Sigma-Aldrich.

Carlo Erba and Merck's provided trifluoroacetic acid (Carlo Erba, CAS=76-05-1) and acetonitrile (Merck, CAS=75- 05-8). The water used in the study was purified with the Water Pro BT Purification System device of LABCONCO (Kansas City, MO, USA).

Sample preparation and HPLC-UV analysis

The samples were prepared though the dilution of 5 mg of each with 10 mL of ultrapure water with three replicates and then were filtered prior to injection into the HPLC-UV to analyze apamin, melittin, and PAL2 contents. The HPLC analysis was carried out using the method by Kokot & Matysiak (2009).

A Supelcosil lc-318 5 µm. 4.6×250 mm column

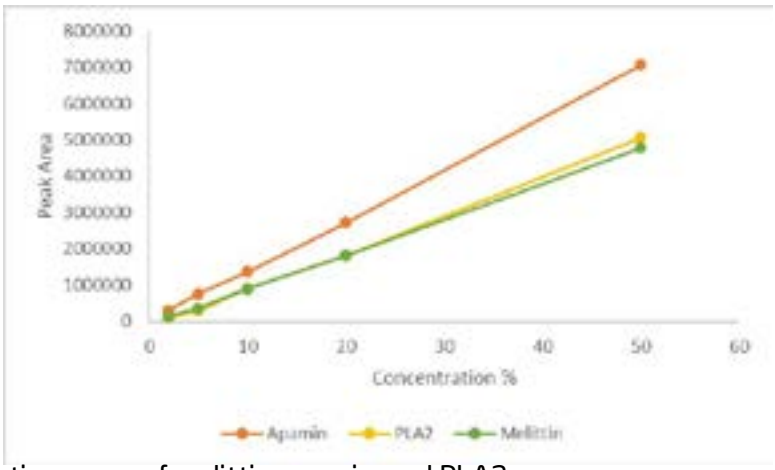


Fig. 1. Standard calibration curve of melittin, apamin, and PLA2.

(Supelcosil HPLC Products) and a linear gradient 5% B – 80% B at 30 min (eluent A – 0.1% TFA in water; eluent B – 0.1 % TFA in acetonitrile: water (80:20)) were used. The mobile phase flow rate was 1 mL/min with a 40 µL of injection volume at 25°C column temperature. The analysis was monitored at 220 nm. The HITACHI HPLC system consisted of a quaternary 5160 pump, a 5260-auto sampler, a 5410 UV detector, and a 5310-column oven. The instrumentation, data acquisition and data reporting control were performed with the use of Chromaster computer software. The standard calibration curve equations were used to calculate the concentration of constituents (Fig. 1) (Samancı & Kekeçoğlu, 2019). The limit of detection (LOD) values were determined for melittin, fosfolipaz A2 and apamin as 3.5%, 2.0% and 0.5% and limit of quantitation (LOQ) values as 1.2%, 8.1% and 2.0%, respectively. The standard calibration curve equation was calculated for melittin (y=97131x-90091; R2=0,9996), apamin (y=140666x-9362,3; R2=0,9995) and PLA2 (y=104057x-177110; R2=0,9985).

Statistical Analysis

The Student's t-test was run to analyze the differences between samples either harvested day- or night-time and either from the inside or entrance of the beehives. Differences in samples relevant to seasons and geographic locations were analyzed separately using one-way ANOVA/independent samples t-tests. Tukey's HSD test was used to establish significant differences between pairs of means after the one-way ANOVA was performed ($P<0.05$) in groups. The SPSS 25.00 for Windows software package was used for the statistical analysis.

RESULTS

The results of these groups based on harvesting time (day or night) and collecting site of the

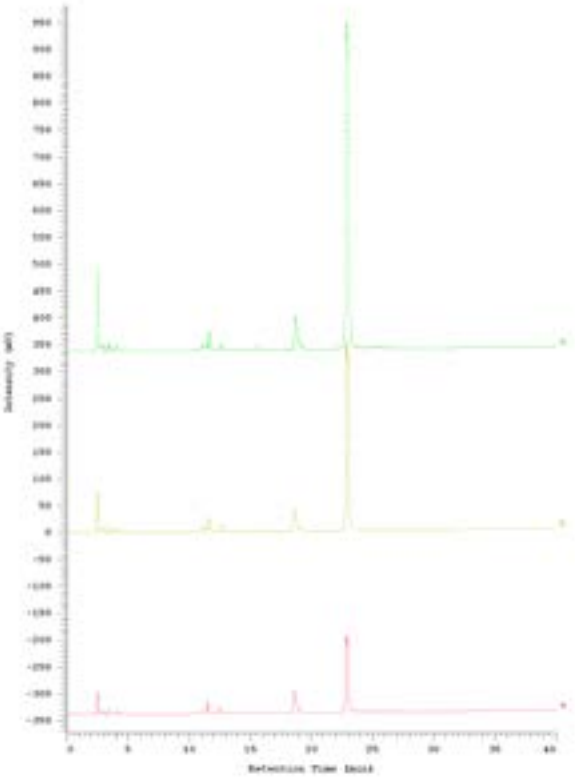


Fig. 2. HPLC chromatograms of different quality HBV samples. A (lower value): Apamin; 1.47%, melittin 20.73%, PLA2, 10.28%. B (average value): Apamin; 1,76%, melittin; 48.66%, PLA2; 10.96%. C (higher value): Apamin; 2.05%, melittin; 62.80%, PLA2; 13.90%.

Table 2.

Apamin, melittin, and PLA2 contents of samples grouped into the factors of harvesting time and collection site of the beehive [a]

	Component	Grouping Factors	Group Code [b]	N	Mean	SE[c]	Min.	Max.	t	p[d]
Harvesting Time	Apamin	Samples collected from the inside of beehives either at day or night-time	IH/DC	16	2.23	0.13	1.62	3.81	0.40	0.68
			IH/NC	17	2.17	0.09	1.62	2.77		
	Melittin		IH/DC	16	39.02	1.94	29.50	61.90	0.13	0.72
			IH/NC	17	39.88	1.44	29.66	48.56		
	PLA2		IH/DC	16	15.79	0.93	10.88	26.25	0.31	0.20
			IH/NC	17	14.35	0.62	10.41	17.86		
Harvesting Site	Apamin	Samples collected at day-time either from the inside or entrance of beehives.	IH/DC	16	2.23	0.13	1.62	3.81	0.40	0.62
			EH/DC	17	2.13	0.16	1.27	3.95		
	Melittin		IH/DC	16	39.02	1.94	29.50	61.90	0.76	0.46
			EH/DC	17	41.25	2.22	25.57	63.18		
	PLA2		IH/DC	16	15.79	0.93	10.88	26.25	0.08	0.11
			EH/DC	17	13.66	0.89	8.49	24.95		

[a] The values represent the mean of three runs of each sample on HPLC.
[b] IH/DC=Harvested from the inside of hive at day-time, IH/NC=Harvested from the inside of hive at night-time, EH/DC=Harvested at the entrance site of hive at day-time, PLA2: Phospholipase A2.
[c] SE: Standard Error
[d] p: Probability of Student test t statistic.

Table 3.

Apamin, melittin, and PLA2 contents of samples (DS) grouped according to the production season [a]

	APAMIN, %					MELITTIN, %				PLA2, %[b]			
SEASON	N	Mean	SE[b]	Min.	Max.	Mean	SE[b]	Min.	Max.	Mean	SE[b]	Min.	Max.
MAY	15	2.00	0.10	1.47	2.77	38.64	1.49	30.96	49.03	14.09	0.62	10.31	17.86
JUNE	18	2.22	0.17	1.27	3.95	38.85	2.43	25.57	63.18	15.19	1.08	8.49	26.25
JULY	10	2.31	0.11	1.73	2.85	41.41	1.66	31.12	47.48	15.44	0.86	10.41	20.95
AUGUST	7	2.23	0.11	1.69	2.61	44.37	1.84	37.37	51.85	12.79	0.70	9.96	14.86
F			0.86				1.17				1.18		
p[c]			0.47				0.33				0.33		
Average value			2.17				40.07				14.58		

[a] The values in the table represent the mean of 3 runs of each sample on HPLC
[b] PLA2=Phospholipase A_2; SE=Standard Error
[c] Probability of F statistic from ANOVA

beehive (entrance or the inside of the hive) are presented in Tab. 2, and the chromatograms of apamin, melittin and PLA2 for selected samples are provided in Fig. 2. The harvesting time of HBV samples, either at day or nighttime, did not significantly affect the amounts of apamin (2.23%-2.17%), melittin (39.02%-39.88%), or PLA2 (15.79%-13.66%) (Tab. 2). Similarly, the collection site of the HBV samples from the beehives, either during the day or nighttime, resulted in no significant differences in the amounts of apamin (2.33%-2.13%), melittin (39.02%-41.25%), and PLA2 (15.79%-13.66%) as presented in Tab. 2 (P>0.05).

The selected protein and peptide content of HBV samples produced in different months are

Table 4.

Apamin, melittin, and PLA2 contents of samples (DGL) obtained from different geographic locations [a]

		APAMIN, %				MELITTIN, %				PLA2, %[b]			
LOCATION	N	Mean	SE[b]	Min.	Max.	Mean	SE[b]	Min.	Max.	Mean	SE[b]	Min.	Max.
DÜZCE	10	2.56	0.16	2.04	3.81	51.87	3.06	39.28	64.04	18.45	1.21	12.70	26.25
MANİSA	5	1.96	0.11	1.74	2.37	37.83	1.51	34.38	42.05	11.12	0.55	9.84	13.00
MUĞLA	5	2.17	0.24	1.47	2.81	36.71	4.54	19.61	43.72	11.64	1.34	7.22	15.48
BALIKESİR	4	1.92	0.33	1.50	2.29	46.97	4.85	41.16	61.47	16.49	3.97	11.47	28.18
MERSİN	3	1.57	0.30	1.28	1.89	44.85	4.44	37.75	53.02	21.26	1.62	18.21	23.75
Total	27	2.17	0.53	1.28	3.81	44.96	1.99	19.61	64.04	15.85	1.02	7.22	28.18
F				4.08				3.51				5.18	
p[c]				0.013				0.023				0.004	

[a] The values in the table represent the mean of 3 runs of each sample on HPLC
[b] PLA2=Phospholipase A_2; SE=Standard Error
[c] Probability of F statistic from ANOVA

presented in Tab. 3. Accordingly, harvesting in four subsequent months from May to August did not significantly influence the amounts of apamin (2.00%-2.31%), melittin (38.64%-44.37%) and PLA2 (12.79%-15.44%) in HBV samples (P>0.05) (Tab. 3).

Tab. 4. Represents the apamin, melittin and PLA2 content of twenty seven HBV samples obtained from beekeepers in Manisa, Muğla, Balikesir, Düzce and Mersin. The statistical analyses revealed significant differences in the amounts of apamin (1.57%-2.56%), melittin (36.71%-51.87%) and PLA2 (11.12%-21.26%), according to the geographic locations (p<0.05). The samples produced in the DAGEM apiary had the highest amounts of apamin and melittin but the second-largest value for PLA2 than other samples (Tab. 4).

DISCUSSION

Three components of HBV, Melittin, Apamin, and PLA2, are considered important quality indicators for their essential bioactivities suitable for apitherapeutic or cosmetics purposes (Banks & Shipolini 1986). Among these constituents, melittin has been reported to be the major component of bee venom ranging between 40-50%, as reported by many studies (Ali, 2012; Banks & Shipolini, 1986; Bogdanov, 2015; Kye-Sung & Ki-Rok, 2009; Moreno & Giralt, 2015; Zhou et al., 2010; Samancı & Kekeçoğlu, 2019). On the other hand, some studies proclaimed much higher or lower values of melittin. For example, Polish scientists noted a value higher than 70% (Rybak-Chmielewska & Szczęsna, 2004) in their HBV samples. However, Romanian scientists revealed a low level of melittin, about 27%, in their samples harvested from the inside of beehives (Ionete et al., 2013). In our study, the average 40% melittin amount in samples harvested from either inside or at the entrance of beehives during the daytime is consistent with the values reported in the literature.

The protein, PLA2, is the second-largest component of honeybee venom as reported to be in the ranges of 10-12% (Banks & Shipolini, 1986; Bogdanov, 2015; Moreno & Giralt, 2015). However, there are also published scientific data showing its amount to be as low as 7% (Kokot & Matysiak, 2009) or as high as 23% (Ionete et al., 2013). In the present study, the average PLA2 values ranging between 14% and 16% in samples harvested either from both sites of the hives in the daytime (Tab. 2) are higher than these reported ranges for Anatolian HBV.

The apamin content of HBV is much lower than the other two significant components, as reported between 2 and 3% by many scientific studies (Banks & Shipolini, 1986; Bogdanov,

2015; Moreno & Giralt, 2015). In the current literature, the highest apamin value is 5.32% (Ionete et al., 2013), and the lowest is 1.75% (Kokot & Matysiak, 2009). According to our data presented in Tab. 2, the apamin amount averaged from 2.13% to 2.23% for Anatolian HBV is consistent with the available information in the literature, while it reaches a maximum (3.95%) in June (Tab. 3).

Collectors located inside the beehive can be used at the bottom of the hive (Han et al., 2007) or to cover the top of the hive (Robson, 1988). Such devices are capable of collecting higher amounts of venom than hive-entrance collectors, as they may be in contact with larger numbers of bees while operating. The collectors located at the base from the apparatus placed in different parts in the hive are exposed to contamination with bees' wastes, therefore they are not recommended. On the other hand, those on the hive or in the form of a frame have a lower risk of contamination (Robson, 1988; Serrinha et al., 2019). According to our research, there is no specific study concerning the inside or entrance of the hive site effect on HBV composition. Most of the studies focus on the amount of the production as a result of site differences. In our study, we did not determine any statistical differences between the harvesting site methods through HBV composition. However, we offer the inside hive collection method due to its effect on production amount and sterilization according to the previous studies.

There have been various studies on the seasons' influence on bee venom protein and peptide components. Owen & Praf (1995) with the dissection method studied HBV melittin content of in connection with seasonal changes and determined that, melittin levels were lower in mid-August than early June. Rybak-Chmielewska & Szczęsna (2004) found that the melittin component differed significantly among HBV samples harvested in different seasons. Ferreira Junior et al. (2010) harvested the bee venom at the entrance of the hives and determined that seasonal changes influence the melittin and PLA2 without a specific association to any climatic parameter.

In contrast with those findings, our results revealed that the seasons had no statistically significant effect on the apamin, melittin and PLA2 components of Anatolian HBV (Tab. 3). However, the highest values for apamin and PLA2 were observed in samples harvested in July and for melittin in August (Tab. 3). Disagreement between previous studies and our results on seasonal effects may be associated with either different harvesting methods or different locations offering different nectar and pollen sources, since venom quality may vary according to the food they consume (Abusabbah et al., 2016; Nowar, 2016).

The study results on locational differences (using the same production method) revealed significant variations in samples collected from five regions in Turkey (Tab. 4). Those locations represent the climate variations as Balıkesir and Manisa are located in the Aegean region, Düzce on the Black Sea and Mersin in the Mediterranean area. In general, the highest mean values for apamin and melittin were observed in samples produced by DAGEM (Düzce University Beekeeping Research and Development Center) Apiary under the controlled conditions of Düzce University. Those sampling locations represent the Black Sea, Aegean and Mediterranean regions with opposing climate parameters and land flora. There may be no direct relationship between the changes in honeybee venom components and climate conditions as described by Ferreira Junior et al. (2010).

Interestingly, the highest reported PLA2 value for Anatolian HBV has been the samples obtained from beekeepers in Muğla have had an average of 28% PLA2 level. Since PLA2 is one of the most effective substances in bee venom content for defense purposes, it may have developed as a self-defense mechanism for the *A. m. anatoliaca* populations against local enemies. However, these differences might also be related to beekeepers' improper practices during harvesting and storing, as the most critical parameters for determining HBV quality. In light of all the data obtained from the study, the composition of honey bee venom has been revealed to be unaffected by neither the

production time nor harvesting site of the hive, but is affected by geographic locations.
However, there is still a need for more research to clarify the location's effect on Anatolian HBV quality. This study presented data to be useful for a proposal leading to a standardized production as HBV is a crucial apiculture products due to its pharmaceutical value. As the bee venom market continues to evolve, imitation and adulteration attempts will be a safety problem for authorities worldwide. Therefore, we hope that the presented research data will help the authorities establish the specifications and standards regarding HBV production and its analysis.

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