EVALUATION OF THE ANTIBACTERIAL, ANTIBIOFILM, ANTIOXIDANT, AND CYTOTOXIC EFFECTS OF SOME TURKISH HONEYS

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ABSTRACT

The aim of our study was to investigate the antibacterial, antibiofilm, antioxidant and cytotoxic activity of nine Turkish honey samples collected, from different locaties of Turkey. Antibacterial activity was evaluated by an agar well diffusion method and also minimum inhibition concentrations (MICs) was determined by microdilution broth technique. The antibiofilm effect of sub-MIC concentrations of honey samples was analyzed by the microplate biofilm assay. The antimicrobial and antibiofilm activity were screened using by six Gram-positive bacteria; *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MU 40), *Staphylococcus epidermidis* (MU 30), *Streptococcus mutans* (ATCC 35668) and *Listeria monocytogenes* (ATCC 7644). The cytotoxicity assays carried out with the brine shrimp lethality bioassay method. Antioxidant capacities were determined in terms of their antiradical capacity using the stable free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH). The honey samples showed the highest antibacterial activity against to *Staphylococcus aureus* and *Bacillus subtilis*. *Staphylococcus epidermidis* and *Listeria monocytogenes* were both moderately sensitive to honey antimicrobial activity. All honey samples showed significant antibiofilm activity. 500 and 375 mg/ml concentrations of honey samples showed considerable activity against *Listeria monocytogenes* biofilms. All the honey samples were found to have potential antioxidant activity having IC_{50} values ranging from 38,75-123,5 mg/ml. In the cytotoxic activity tests, LC_{50} values were obtained in the range of 15,88-163899. The results of the present investigation suggest that most of the

studied honey are potentially good source of antibacterial, antibiofilm, antioxidant and anticancer agents.

Keywords: Honey, antibacterial, antioxidant, antibiofilm, cytotoxic effect

Introduction

Honey is a sweet and flavorful product which has been consumed as a high nutritive value food. It is essentially composed of a complex mixture of carbohydrates (of which fructose and glucose account for nearly 85–95%) and other minor substances, such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (White, 1975). Research indicates that honey have functional properties in human health promotion which depend largely on the floral source of the honey. These properties could be associated to honey high osmolarity and antibacterial properties (Effem, 1988). The major antibacterial properties are related to the level of hydrogen peroxide determined by relative levels of glucose oxidase and catalase (Weston, 2000).

Although the honey therapeutic action has been taken some attention by researchers, studies only have been done on screening the raw honey samples on antimicrobial activity (Taormina et al., 2001; Al-Mamary et al., 2002; Kucuk et al., 2007; Basualdo et al., 2007; Estevinho et al., 2008; Truchado et al., 2009; Alvarez-Suarez et al., 2010; Gomes et al., 2010; Silici et al, 2010) and on antioxidant capacity (Rauha et al., 2000; Frankel et al., 1998; Estevinho et al., 2008; Alvarez-Suarez et al., 2010; Silici et al, 2010). To our knowledge, there are limited studies on the antibiofilm (Badet and Quero 2011) and cytotoxic activity (Jaganathan et al., 2010) of the honeys. The aims of the present study were to contribute to increase the current scarce knowledge about the cytotoxic and antibiofilm activity of nine Turkish honeys as well as their antioxidant and antimicrobial activity.

Material and Methods

Honey samples

A total nine samples of handicraft honeys, from different locations of Turkey, were directly collected through beekeepers (Table 1).

Table 1. Geographical origin of honey samples studied.

Sample code	Region of Turkey	Type
B-1	Nevsehir	Pumpkin
B-2	Zonguldak	Chestnut
B-3	Muğla-Datca	Thyme
B-4	Gaziantep	Herba euphorbiae
B-5	Muğla- Marmaris	Chaste (Vitex agnus-castus)
B-6	Van-Muradiye	Multifloral
B-7	Muğla-Gökova	Eucalyptus
B-8	Muğla-Bodrum	Honeydew
B-9	Muğla-Milas	Honeydew

Bacterial Strain and Culture Conditions

The bacterial strains used as test organisms were *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MU 40), *Staphylococcus epidermidis* (MU 30), *Streptococcus mutans* (ATCC 35668) and *Listeria monocytogenes* (ATCC 7644). The strains were obtained from the Mugla University Culture Collection. The above-mentioned bacteria, except *S.mutans,* were cultured in nutrient broth (NB) (Difco, USA), *S.mutans* was cultured in Brain Heart Infusion Broth (BHIB)(Difco). The strains were incubated at 37±0.1ºC for 18-24 h. Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 McFarland Standard Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water inoculated on NB, BHIB to check the viability of the preparation. The cultures of microorganisms were maintained in their appropriate agar slants at 4ºC throughout the study and used as stock cultures.

Screening of antimicrobial activity of honey samples.

Antimicrobial activity of honey samples was determined by agar well diffusion method and also minimum inhibition concentrations (MICs) was determined by microdilution broth technique.

Determination of antibacterial activity by the agar-well diffusion method

The assays evaluate the inhibitory activity of four honey concentrations prepared in sterile distilled water: 75%; 50% ; 25% and 12,5% honey (w/v). A suspension of the tested microorganisms (100 µl of a 10^5-10^6 cfu/ml standardized microbial suspension) were plated onto Mueller Hinton agar. Wells (7 mm diameter) were punched in the plates using a sterile stainless steel borer. The wells were filled with 100 µl different honey solutions. The plates were incubated at 37ºC for 24 h. The diameters of the inhibitory zones were measured in milimetres.

Determination of minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) was determined by a microdilution broth method as recommended by the Clinical and Laboratory Standards Institute. The test medium was MHB and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (200µL) were inoculated into the wells of 96-well microtitre plates (Nunc F96 MİKroWell™ plates; NunclonTM Δ, Denmark) in the presence of honey solutions with different final concentrations (12.5,25,37.5,50 mg/ml). All microplates were incubated at 37 $^{\circ}$C for 24 h. The MIC was defined as the lowest concentration of essential oil at which no visible growth was observed. The optical density at 550 nm (OD_{550}) of each well content was recorded using an microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland), as a measure of microbial growth. Each assay was performed in triplicate for all bacteria.

Microplate biofilm assay

The effect of subinhibitory concentrations of honey samples on the biofilm formation of bacterial pathogens was determined by quantifying the biofilm biomass through microplate biofilm assay. Briefly, 1% of overnight cultures (OD adjusted to 0.4 at 600 nm) of test pathogens were added into 200 µl of fresh TSB medium and cultivated in the presence and absence of honey solutions (6.25-50 mg/ml) without agitation for 48 h at 37 ºC. The wells containing only TSB served as control. After incubation, the wells were washed with water to

remove planktonic bacteria. The remaining bacteria were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution that is not specifically staining the adherent bacteria. Microplates inverted and vigorously tap on paper towels to remove any excess liquid and air dried. Further, dried plates were washed with 200 µl of 33% glacial acetic acid (Sigma Chemical Co). Biofilm stains solubilized at room temperature. After shaking and pipetting of wells, 125 µl of the solution from each well transferred to a sterile tube and volume reached to 1 ml with distilled water. Finally optical density of each well measured at wavelength of 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Each strain was tested for biofilm production in duplicate and the assay was repeated three times. Replicate absorbance readings for each concentration tested were averaged and the average of the media control was subtracted. This value was then divided by the mean absorbance of the (cell+TSB) and multiplied by 100.

Antioxidant assay to determine DPPH scavenging activity

The hydrogen atom or electron donation abilities of the corresponding extracts were measured from the bleaching of the purple-coloured ethanol solution of DPPH. This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits and Bucar, 2000; Cuendet et al., 1997). Different concentrations of the honey solutions (200 ml) in ethanol were added into 5 ml of a 0.004% (w/v) ethanol solution of DPPH. After 30 min incubation period at room temperature, the absorbance was measured against a blank at 517 nm. Inhibiton of free radical by DPPH in percent (I%) was calculated using the following expression:

$$I\% = (A_{Blank} - A_{sample} / A_{blank}) x\ 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compund. Honey concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagent BHT and alpha tocoferol were used as a positive control and all tests were carried out in triplicate.

Brine shrimp lethality bioassay

The cytotoxic activity of the honey samples were evaluated using Brine shrimp lethality bioassay method where 3 graded doses (viz 1000 µg/mL, 100 µg/mL and 10 µg/mL) were used (Meyer et al., 1982). Brine shrimps (Artemia salina Leach) nauplii (Ocean 90, USA) were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 h. The mature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. Vincristine sulfate was used as a reference standard in this case. The numbers of survivors were counted after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The larvae did not receive food. To ensure that the mortality observed in the bioassay could

be attributed to bioactive compounds and not to starvation; we compared the dead larvae in each treatment to the dead larvae in the control.

Statistical analysis

The median lethal concentration (LC_{50}) and 95% confidence intervals of the test samples were calculated using the probit analysis method described by Finney (1971), as the measure of toxicity of the honey solutions..

Results and Discussion

The results of the antibacterial investigations using the agar-well diffusion method are given in Table 2. It indicates that different bacterial species demonstrated different levels of sensitivities towards the tested samples of honeys.The diameter for zone of inhibition for honey samples ranged from 34 to 9 mm at various concentrations used. Maximum inhibitory effect was towards on *B. subtilis* and minimum inhibitory effect was towards on *S.mutans* and *L.monocytogenes*.

Table 2. Antibacterial activity of honeys using agar-well diffusion method

	Honey samples									**Antibiotics**	
	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	P	AM
Test bacteria	Inhibition zone diameter (mm)										
B. subtilis **ATCC 6633**	32	31	31	30	24	34	26	30	30	32	17
S. aureus **ATCC 25923**	-	15	20	13	22	14	12	19	15	31	17
S. aureus **MU 40**	-	10	-	-	24	14	-	9	10	13	11
S. epidermidis **MU 30**	-	9	-	-	14	16	10	12	9	14	10
S. mutans **ATCC 35668**	-	-	-	-	-	18	-	-	-	20	12
L. monocytogenes **ATCC 7644**	-	-	-	-	-	14	-	-	-	29	18

-:No inhibition zone, P: Penicilline (10 IU), AM:Ampicillin (10 mcg)

The results are given in the table only 75% honey concentrations.

Results pertaining to the antibacterial investigations using the microdilution broth technique are given in Table 3. MIC values obtained 12.5-50 mg/ml against tested bacteria. The honey samples showed highest antibacterial activity against *B.subtilis* and *S.aureus* ATCC 25923. In addition, *S.aureus* MU40 and *S.epidermidis* MU 30 were moderately sensitive to the antimicrobial activity of B-2, B-5, B-6, B-8 and B-9 honeys. However *S.mutans* and *L.monocytogenes* were the most resistant microorganisms. The high antibacterial activity against all bacteria tested in B-6 (Van-Muradiye, multifloral honey) were determined.

Table 3. Minimal inhibitory concentration (MIC) values of honeys

Test bacteria	Honey samples									Antibiotics	
	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	AM	OX
	MIC (mg/ml)										
B. subtilis ATCC 6633	25	25	25	25	37.5	12.5	37.5	25	25	0,156	0,156
S. aureus ATCC 25923	-	50	37.5	50	37.5	50	50	37.5	50	0,156	0,156
S. aureus MU 40	-	50	-	-	37.5	37.5	-	50	50	0,625	0,156
S. epidermidis MU 30	-	-	-	-	50	37.5	-	37.5	50	0,312	0,078
S. mutans ATCC 35668	-	-	-	-	-	37.5	-	-	-	0,156	0,312
L. monocytogenes ATCC 7644	-	-	-	-	-	50	-	-	-	0,625	0,625

-: No inhibition, AM: Ampicillin, OX: Oxytetracycline

The inhibitory effect on tested bacteria biofilm formation of the honey solutions is shown Table 4. All honey samples inhibited *B.subtilis* biofilm formation at sub-MIC levels. Also all honey samples, except B-1, reduced *S.aureus* ATCC 25923 biofilm formation at various percentage. *S.aureus* MU 40 and *S.epidermidis* MU 30 biofilm formations are reduced by B-5, B-6 and B-8 honey samples. Only B-6 showed antibiofilm activity against *S.mutans* and *L.monocytogenes.* For *S.mutans*, MBC and MIC were determined as 37.5 mg/mL and 25 mg/mL and biofilm formation is reduced to 27.77% and 21.96% in these concentrations. *L.monocytogenes* biofilm formation has been reduced to 41.05% in MIC and 17.8% in MIC/2.

The results obtained for DPPH radical scavennging activity of these honeys are summarized in Table 5. A high variability was observed in the scavenging honey activity of different floral and honeydew honeys evaluated. The IC50 values of samples using free radical scavenging assay were determined. The lowest value in IC_{50} values was determined in Sample B-6 as 38.75 mg/ml. As it was expected, a higher percentage of radical scavenging capacity was observed in honeydew honeys, in accordance with recent studies that have found that the darkest coloured honeys present the highest antioxidant capacity (McKibben and Engeseth, 2002; Gheldof and Engeseth, 2002; Vela et al., 2007). According to our results, honey samples showed a good correlation between antioxidant capacity and antibacterial/antibiofilm activity.

Table 4. The effect of honeys on tested bacteria biofilm formation expressed as percentage inhibition.

		Honey samples								
	Concentration	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9
Test bacteria	mg/ml	Percentage (%) inhibition								
	37.5	NT	NT	NT	NT	46.02	NT	64.81	NT	NT
B. subtilis	25	53.19	63.62	54.61	44.17	29.13	NT	42.59	71.2	53.35
ATCC 6633	12.5	38.06	30.06	16.38	20.39	6.29	72.25	17.03	40.5	35.8
	6.25	-	-	-	8.15	-	24.47	-	12.15	10.74
	50	NT	31.75	NT	60.82	NT	NT	58.83	47.73	56.45
S. aureus	37.5	NT	26.54	43.26	44.5	56.95	50.23	32.54	27.17	26.3
ATCC 25923	25	NT	7.1	15.7	17.8	29.67	41.74	15.88	3.48	9.2
	12.5	NT	-	-	-	9.79	18.63	5.4	-	-
	50	NT	32.64	NT	NT	NT	NT	NT	53.09	47.41
S. aureus	37.5	NT	25.83	NT	NT	33.67	13.89	NT	30.08	38.7
MU 40	25	NT	8.87	NT	NT	8.16	7.67	NT	8.29	11.61
	12.5	NT	-	NT	NT	-	-	NT	-	-
	50	NT	NT	NT	NT	21.19	NT	NT	NT	49.68
S. epidermidis	37.5	NT	NT	NT	NT	14.84	13.89	NT	37.74	34.02
MU 30	25	NT	NT	NT	NT	6.68	7.67	NT	20.08	17
	12.5	NT	NT	NT	NT	-	-	NT	4.17	6.48
	37.5	NT	NT	NT	NT	NT	27.77	NT	NT	NT
S. mutans	25	NT	NT	NT	NT	NT	21.96	NT	NT	NT
ATCC 35668	12.5	NT	NT	NT	NT	NT	9.3	NT	NT	NT
	6.25	NT	NT	NT	NT	NT	-	NT	NT	NT
	50	NT	NT	NT	NT	NT	41.05	NT	NT	NT
L. monocytogenes	37.5	NT	NT	NT	NT	NT	28.99	NT	NT	NT
ATCC 7644	25	NT	NT	NT	NT	NT	17.8	NT	NT	NT
	12.5	NT	NT	NT	NT	NT	6.23	NT	NT	NT

NT: Not tested, -: No inhibition

Table 5. IC_{50} values[a] (mg/ml) of honey extracts in DPPH scavenging assays

Sample	DPPH (IC_{50} [a])
B-1	75.8
B-2	112.75
B-3	123.5
B-4	110.25
B-5	73.5
B-6	38.75
B-7	94.25
B-8	43.5
B-9	71
BHT	0.48
Alpha-tocoferol	1.75

[a] IC_{50} (mg/ml): effective concentration at which 50% of DPPH radicals are scavenged.

The LC_{50} values of the brine shrimp lethality bioassay obtained for these samples and that of the positive control, Vincristine sulphate, have been presented in Table 6. Three of honey samples (B-2, B-7 and B-9) exhibited significant toxicity towards brine shrimps. The LC_{50} values of these samples were within the range of 15.882 to 86.906 µg/ml, whereas that of the positive control (Vincristine sulphate) was 0.423 µg/ml. So these honey samples can be considered as a promising candidate for a food derived anticancer compound. LC_{50} values obtained by the 4 honey samples (B-1, B-4, B-5, B-8) are classified as non-toxic, the 2 samples (B-3 and B-6) are classified as harmful.

Table 6. Cytotoxic activity of honey samples on brine shrimp nauplii.

Honey number	LC50 (µg/ml)	Solution(ppm)	Number of dead organisms	95% confidence interval	The degree of toxicity
		1000	14		
B-1	LC/EC 50.00	100	12	2025.325	Non-toxic
		10	9		
		1000	19		
B-2	LC/EC 50.00	100	18	15.882	toxic
		10	14		
		1000	18		
B-3	LC/EC 50.00	100	13	466.003	harmful
		10	9		
		1000	11		
B-4	LC/EC 50.00	100	10	163899.703	Non-toxic
		10	6		
		1000	15		
B-5	LC/EC 50.00	100	11	51359.004	Non-toxic
		10	9		
		1000	17		
B-6	LC/EC 50.00	100	13	485.005	harmful
		10	10		
		1000	22		
B-7	LC/EC 50.00	100	14	86.906	toxic
		10	10		
		1000	15		
B-8	LC/EC 50.00	100	6	1346.210	Non-toxic
		10	4		
		1000	21		
B-9	LC/EC 50.00	100	15	59.083	toxic
		10	12		
		1000	30		
Vincristine	LC/EC 50.00	100	27	0.423	Highly toxic
		10	25		

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