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IN VITRO ASSESSMENT OF THE ANTIVIRAL ACTIVITY OF GREEK BEE BREAD AND BEE COLLECTED POLLEN AGAINST ENTEROVIRUS D68

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ABSTRACT

Bee bread is derived from bee collected pollen which is processed by the bee, fermented and stored in the hive. Few studies have assessed the antimicrobial activity of bee bread against major bacterial pathogens and fungi. However, to the best of our knowledge, neither bee collected pollen nor bee bread, have so far been investigated for their antiviral properties. In the present study, 18 Greek bee bread and 2 pollen samples from different botanical sources and geographical locations were assessed regarding their in vitro antiviral activity against Enterovirus D68. A cell culture assay combined with a comparative Real-Time PCR assay was performed using different sample concentrations to assess the antiviral activity. MTT assay was implemented in order to calculate the sample toxicity levels on cell culture. Our data suggest that Greek bee bread and bee collected pollen exhibit potent antiviral activity against EV-D68 with IC_{50} values ranging from 0.048 to 5.45 mg/ml. Greek bee bread and bee collected pollen are promising sources of antiviral compounds and should be further studied against diverse viruses in order to explore their full antiviral potential.

Keywords: Antiviral, Enterovirus, Bee Bread, Pollen, Bee Hive Products

INTRODUCTION

Bee bread (BB) is a honey bee product derived from bee collected pollen (BCP), processed, fermented and stored in the hive. Fermentation of BCP is catalyzed by bee enzymes and microorganisms (bacteria, molds and yeasts), leading to a more nutritious product. BB consists of proteins, carbohydrates, lipids and fatty acids, vitamins, micro- and macro elements and phenolic compounds (**Didaras *et al.*, 2020**). Few studies have demonstrated the antimicrobial activity of BB against major bacterial pathogens and fungi (**Didaras *et al.*, 2020**; **Pełka *et al.*, 2021**). However, the antiviral activity of BB and BCP has not been studied. Lee *et al.* have studied the antiviral activity against *Influenza A* strains of certain BCP compounds of Korean *Papaver rhoeas* (**Lee *et al.*, 2016**). Other bee products, namely honey, royal jelly, propolis and bee venom were investigated regarding their antiviral properties. Furthermore, bee products have been recently tested for their possible use against SARS-CoV -2 (**Abu-Serie & Habashy**, **2019**; **Berretta et al.**, **2020**; **Hood et al.**, **2013**; **Hossain et al.**, **2020**; **Lima et al.**, **2021**; **Uddin et al.**, **2016**).
Enterovirus D68 (EV-D68) belongs to genus *Enterovirus* of the *Picornaviridae* family having characteristics of both the *Enterovirus* (EV) and *Rhinovirus* (RV) species. The genome consists of a 7.500nt single stranded positive sense RNA (+ssRNA). EV-D68 was first isolated and identified in 1962 (**Oberste *et al.*, 2004**). Since then, reports of EV68 isolation have been very uncommon, until 2014 when severe respiratory illness associated with a nationwide outbreak of EV-D68 was reported in the USA (**Midgley *et al.*, 2015**). Most of the infected patients were children and the clinical manifestations varied from mild to severe respiratory symptoms. Furthermore, recent studies have implicated EV-D68 with acute flaccid myelitis (AFM) (**Aliabadi *et al.*, 2016**; **Hu *et al.*, 2019**; **Mishra *et al.*, 2019**).
Against EV-D68 there are no licensed therapies to prevent infection or AFM disease (**Vogt *et al.*, 2020**). Drugs targeting picornaviruses and enteroviruses have been developed but none of them has shown activity against EV-D68 at clinically relevant concentrations (**Oermann *et al.*, 2015**).
In this study, 18 BB and 2 BCP samples from different botanical sources were assessed *in vitro* regarding their antiviral activity against EV-D68.

MATERIALS AND METHODS

Bee bread (BB), bee collected pollen (BCP) samples and virus strain

A total of 18 BB and 2 BCP samples harvested in different regions of Greece between March-October 2019 were provided by beekeepers. Each sample was assigned a unique reference number, and details regarding the botanical source and geographical location were recorded (Table 2). BB samples were directly collected from honeycombs and then stored in sterile plastic containers at -20ºC.
Each one of BB and BCP samples was dissolved in Dulbecco's Modified Eagle cell culture medium (D-MEM, LM-D1113, Biosera, France) for one hour (1 h) at room temperature, centrifuged for 10 min at 10,000 rcf and then the liquid fraction was filtered through a Branchia 0.22μm syringe filter (Labbox Labware, S.L., Barcelona, Spain). The filtered BB and BCP suspension was serially diluted and used for the assays.
Enterovirus D-68, Fermon (California/62) strain (RIVM, The Netherlands), inoculated in Rhabdomyosarcoma (Rd) cells (human rhabdomyosarcoma cell line—CCL-136™, ATTC) was firstly titred calculating the $TCID_{50}$ (**Hierholzer & Killington**, **1996**), and then serially diluted in order to obtain 10^2 $TCID_{50}$/0.1ml virus titre.

Palynological Analysis

Palynological analysis of the 18 BB and 2 BCP samples in order to characterize the botanical origin was conducted by CheMa laboratories (Korinthos, Greece) as described before (**Didaras *et al.*, 2021**). In brief, 5–10 mg of each sample was diluted in 1 mL of deionized water using a vortex mixer and 0.5 mL of each suspension was spread on a 22 × 22 mm area of a microscopy slide and dried at 39 °C. The pollen grains were identified using a Euromex BioBlue optical microscope at 400× magnification. The following databases were used in order to identify the pollen grains: Pollen Atlas, available at pollenatlas.net, the pollen Wiki database and the pollen library at the CheMa laboratories.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

In order to assess the cytotoxicity of BB and BCP samples against Rd cells the MTT colorimetric assay used (van Meerloo et al, 2011). Rd cells in D-MEM supplemented with 2% fetal bovine serum (FB-1001, Biosera) were seeded in 96 well plates (Biologix Europe, Germany). Cells were incubated for 24 hours at 37ºC thus forming a monolayer. After that, the medium was removed and 100μl of each BB or BCP dilution in D-MEM medium containing were added on the 96 well plate in triplicates. The final concentrations of tested BB and BCP samples ranged from 16 to 0.0625 mg/ml (two-fold dilutions). A cell control containing only Rd cells was used in triplicate as a negative control. The plate was incubated at 37ºC for 24 h. After the incubation 10 μl of the MTT (final concentration 0.5 mg/ml) added to each well. The plate was incubated at 37ºC for 1 h and 100 μl MTT solvent (10% SDS and 0.01 M HCl) added for the solubilization of the formazan crystals. The cytotoxicity effect assessed by spectrophotometric measure at 570 nm.

Antiviral activity assay based on a cell culture method

In order to assess the antiviral activity of BB and BCP samples against EV-D68, a cell culture assay was implemented. Rd cells in D-MEM supplemented with 2% fetal bovine serum (FB-1001, Biosera) were seeded in 96 well plates (Biologix Europe, Germany). Cells were incubated for 24 hours at 37ºC thus forming a monolayer. After that, the medium was removed and 200μl of each BB or BCP dilution in D-MEM medium containing 100μl EV-D68 virus were added on the 96 well plate in triplicates. The final concentrations of tested BB and BCP samples ranged from 16 to 0.0625 mg/ml (two-fold dilutions). The plate was incubated at 37ºC and examined daily for development of Cytopathic Effect (CPE).
BB and BCP control wells containing Rd cells and BB or BCP samples served as toxicity controls, in order to differentiate CPE due to virus activity or toxicity exerted by the sample itself. Each concentration was tested in triplicates.
A cell control containing only Rd cells was used in triplicate as a negative control. For a valid test, cell control should have an intact monolayer of Rd cells. A virus control containing Rd cells and EV-D68 was used as a positive control in triplicate. When 100% CPE was observed in virus control then the assay was ended and 96 well plates were stored at -20ºC till further analysis.

RNA Extraction and Reverse Transcription

Every single BB/BCP sample was tested in triplicate pooled in a 1.5 ml tube. RNA extraction was performed using 100 μl of each tube according to Casas *et al.*, 1995 based on the chaotropic agent guanidine thiocyanate. At the end of this protocol the pellet was dried and dissolved in 100μl of sterile double-distilled, DNase-RNase free, water (DEMO S.A, Athens, Greece).
In order to detect via Real-Time PCR, the positive sense ssRNA viral genome of EV-D68, a Reverse Transcription assay was performed using random primers (Macrogen, South Korea) and FastGene Scriptase II (Nippon Genetics, Japan) according to manufacturer's protocol. The synthesized cDNA was stored at -20ºC till further use.

Comparative Real-Time PCR assay

Antiviral activity of samples was assessed by a comparative Real-Time PCR assay, calculating the relative concentration of viral titre in cell cultures containing different BB/BCP concentrations compared to the viral titre used as positive control.
Three microliters of cDNAs were added in a 0.2ml tube containing 1X FastGene Mix (Nippon Genetics, Japan), 50nM ROX Low, 10pmol of each universal enterovirus primer targeting the 5'-UTR region EV2 (5'-CCCCTGAATGCGGCTAATC-3'),
EV1 (5'-GATTGTCACCATAAGCAGC-3') (**Monpoeho *et al.*, 2000**) and ddH_2O up to 20μl.
The Comparative Real-Time PCR was performed at Eco48 (PCRMax) instrument at the following protocol: 95ºC for 2 minutes, 40 cycles of 95ºC for 5 seconds and 60ºC for 30 seconds followed by a melting curve analysis step. Cycle threshold (Ct) values were used to calculate the relative concentration as means of $2^{-\Delta Ct\,(Control-Sample)}$, according to EcoStudy (PCRMax).

CC_{50}, IC_{50} and SI calculation

Cytotoxicity concentration 50% (CC_{50}) is defined as the concentration of a substance that will kill half the cells in an uninfected cell culture (**Pritchett *et al.*, 2014**). Cell toxicity of BB and BCP was calculated using the MTT assay (**van Meerloo *et al.*, 2011**).
The half maximal inhibition concentration (IC_{50}) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function (**Pritchett *et al.*, 2014**). In our study IC_{50} was used to measure the antiviral potency of BB and BCP samples against EV-D68. More specifically IC_{50} value expresses the concentration of each BB sample that corresponds to 50% decrease of the viral titre and calculated via the Real-Time PCR results.

Finally, Selectivity Index (SI) is a ratio that measures the window between cytotoxicity and antiviral activity by dividing the given CC_{50} value into the IC_{50} value (CC_{50}/IC_{50}) (**Cavalli *et al.*, 2012**).

Statistical Analysis

Correlation analysis was conducted using Spearman′s correlation analysis. Values of $p < 0.05$ were considered to indicate statistically significant differences. Statistical analysis was performed using the SPSS version 13.0 statistical package (SPSS, Inc., Chi- cago, IL, USA).

RESULTS AND DISCUSSION

Several bee products, namely honey (**Shahzad & Cohrs**, **2015**; **Wan Yusuf *et al.*, 2019**; **Watanabe *et al.*, 2014**), propolis (**Gekker *et al.*, 2005**; **Güler *et al.*, 2021**; **Maruta & He**, **2020**; **Shimizu *et al.*, 2008**), bee venom (**Memariani et al.**, **2020**; **Uddin et al.**, **2016**), royal jelly (**Habashy & Abu-Serie**, **2019**, **2020**) and even beeswax (**Hassan *et al.*, 2015**) exert significant antiviral activity. Since the outbreak of Covid-19 many published reviews focus on the antiviral activities of bee products and their potential applications against the pandemic (**Al-Hatamleh *et al.*, 2020**; **Berretta *et al.*, 2020**; **Lima *et al.*, 2021**). Surprisingly, neither BCP nor BB, have so far been investigated for their antiviral properties. Nevertheless, both BCP and BB contain a plethora of polyphenols which are renowned for their potent antiviral activity (**Chen *et al.*, 2006**; **Yi *et al.*, 2004**). In this study, 18 BB and 2 BCP samples were assessed *in vitro* against Enterovirus D-68 strain.

Palynological Analysis

In a recent study of our group (**Didaras *et al.*, 2021**), palynological analysis was performed and presented for the 18 BB samples tested in this study. Moreover, in this study, palynological analysis was performed for the 2 BCP samples to investigate whether there is any correlation between the exerted cytotoxicity, antiviral activity and botanical origin. Table 1 demonstrates the pollen grain content (%) of each BCP sample.

Table 1 Harvest period and palynologic analysis of BCP samples.

		BCP Sample	
		19	**20**
Harvest Period		Autumn 2019	Spring 2019
Pollen Family	Amaranthaceae	17.7	-
	Araliaceae	57.9	-
	Asteracea	-	0.2
	Boraginaceae	4.3	-
	Brassicaceae	-	7.1
	Cistaceae	0.5	-
	Cupressaceae	3.6	-
	Fabaceae	4.9	19.9
	Guttiferae	-	27.5
	Oleaceae	-	2.2
	Rosaceae	9.5	15.8
	Saliaceae	-	24.4
	Scrophulariaceae	-	1.2
	Unknown	1.3	1.6
	Zygophylaceae	0.3	-

MTT assay and CC_{50} values

Employment of MTT assay has determined the toxicity levels of BB and BCP samples on Rd cells. As shown in Table 2, the toxicity levels expressed as CC_{50} values, range from 0.17 to 10.19 mg/ml, indicating that high concentrations of BB and BCP are harmful to Rd cells. These levels are relatively high compared to cytotoxicity exerted by honey on MDCK cells (**Watanabe *et al.*, 2014**). Nevertheless, other bee hive products show similar cytotoxicity levels in various cell lines. For example, Hatay propolis samples showed cytotoxic effects on Hep 2 cells at levels of 0.2 μg/mL (**Yildirim *et al.*, 2016**). However, larger number of samples should be tested on Rd as well as other cell lines (f.i. MDCK, Vero and MRC-5) in order to safely conclude on BB/BCP cytotoxicity.

Real-Time PCR and IC_{50} values

A comparative Real-time PCR assay was developed to monitor the viral copy number in the presence of tested samples. We hypothesized that, exerted antiviral activity of BB or BCP samples should lead to a decrease in EV-D68 genome copy number compared to virus control after co-inoculation of virus and samples. This

assay targets the highly conserved 5'UTR region, a region that is widely used for the molecular detection of enteroviruses (**Monpoeho *et al.*, 2000**).
Employment of the comparative Real-time PCR assay has demonstrated a decreasing copy number of EV-D68 when the virus is co-inoculated with BB or BCP samples in cell culture. These data suggest that all BB and BCP samples exert antiviral activity in a concentration-depended mode. The antiviral activity is expressed as IC_{50} values (Table 2) ranging from 0.048 to 5.45 mg/ml.
IC_{50} values are highly variable (over 100-fold) suggesting that the antiviral effect could heavily depend on botanic source and geographic location in accordance to the antibacterial activity exerted by honey (**Stagos *et al.*, 2018**)

Table 2 Geographic location, botanical source and antiviral profile (CC_{50}, IC_{50} and SI values) of BB and BCP samples.

Sample		Location	Botanical Source	(mg/ml)		SI
				CC_{50}	IC_{50}	
Beebread	1	Larissa	Multifloral	4.73 ± 0.02	0.048	98.542
	2	Thessaly	Multifloral	2.32 ± 0.17	0.31	7.484
	3	Mount Pelion	Multifloral	0.23 ± 0.4	0.42	0.548
	4	Larissa	Multifloral	8.60 ± 0.04	0.11	78.182
	5	Mount Pelion	Hedera	6.83 ± 0.09	0.33	20.697
	6	Thessaly	Multifloral	0.17 ± 0.03	0.53	0.321
	7	Heraklion	Multifloral	1.28 ± 0.06	0.052	24.615
	8	Kozani	Multifloral	0.63 ± 0.02	0.091	6.923
	9	Rethymnon	Multifloral	0.31 ± 0.02	0.2	1.550
	10	Arta	Multifloral	5.25 ± 0.07	1.003	5.234
	11	Chania	Multifloral	3.78 ± 0.02	0.182	20.769
	12	Larissa	Multifloral	1.03 ± 0.05	0.28	3.679
	13	Larissa	Cistus	0.52 ± 0.02	0.087	5.977
	14	Lasithi	Multifloral	3.87 ± 0.04	0.5	7.740
	15	Evoia	Multifloral	4.15 ± 0.06	0.58	7.155
	16	Peloponnese	Multifloral	7.56 ± 0.02	0.13	58.154
	17	Chalkidiki	Multifloral	8.03 ± 0.01	0.34	23.618
	18	Mount Athos	Castanea	0.32 ± 0.05	5.45	0.059
Pollen	19	Mount Pelion	Hedera	4.85 ± 0.01	0.91	5.330
	20	Thessaly	Multifloral	10.19 ± 0.02	0.27	37.741

Selectivity Index

Selectivity Index (SI) values have been calculated for each sample, taking into account both CC_{50} and IC_{50} values. The higher the SI ratio, the more effective and safer a substance would be (**Cavalli *et al.*, 2012**). As shown in Table 2, SI values are highly variable (1,670 –fold) ranging from 0,059 to 98,542.
Three (3, 6 and 18) out of twenty samples (15%) demonstrated SI value lower than 1, meaning that viral inhibition is observed in concentrations which are toxic for Rd cells. The other 17 samples (85%) demonstrated SI value higher than 1 meaning that viral inhibition is observed in concentrations that are not toxic. Interestingly, 8 samples (40%) demonstrated SI values higher than 20 (No 1>4>16>20>7>17>11>5), meaning that the IC_{50} concentration is more than 20 times smaller than the correspondent CC_{50} concentration, an indication of potent antiviral effect.

Correlation Analysis

BCP is fermented to BB, a process that includes bees, plants and the microbiome. Each partner might be a source of antiviral compounds such as phytochemicals (**Didaras *et al.*, 2020**; **Ha *et al.*, 2020**), bee secretions (**Habashy & Abu-Serie, 2020**) and secondary metabolites (**Esawy *et al.*, 2011**; **Zhang *et al.*, 2015**) that presumably contribute to the antiviral activity.
In order to elucidate the role of plants, the botanical origin of the samples was taken into account. Statistical analysis was performed in order to assess the correlation among the CC_{50}, IC_{50} and SI values and the major pollen families present in each BB and BCP sample. Only the dominant (> 10%) pollen families were included. Moreover, pollen families that were present in less than three samples were excluded from further analysis.

A negative strong correlation between IC_{50} values and Brassicaceae pollen content was observed (r=0.90; p<0.05). This correlation indicates that compounds present in Brassicaceae pollen might be responsible for the antiviral activity of these samples though further investigation of this hypothesis is needed. Interestingly, a strongly positive and statistically significant correlation between CC_{50} values and pollen content was not observed but a strong positive correlation (r=0.90; p<0.05) was observed between Rosaceae pollen content and SI values (Table 3).
Brassicaceae family, also known as the mustard family, includes plants used in traditional medicine such as *Sinapis nigra*, and *Sinapis alba* as well as cultivated plants with high antioxidant potency like cabbage, broccoli, and cauliflower (**Didaras *et al.*, 2021**). On the other hand, Rosaceae family includes many fruit plants. Future research will reveal whether the pollen of certain Brassicaceae and Rosaceae species detected in BB might contain potent antiviral compounds.

Table 3 Correlation coefficient (r) values estimated from correlation analysis of CC_{50}, IC_{50} and SI values against dominant families of pollen content in each sample.

Pollen Family	IC_{50}	CC_{50}	SI
Araliaceae	1,000**	0.500	-0.500
Boraginaceae	0.500	-1,000**	-0.500
Brasicaceae	-,900*	0.600	0.600
Cistaceae	-0.400	0.200	0.400
Ericaceae	1,000**	0.500	-0.500
Fabaceae	0.095	0.238	0.048
Fagaceae	0.200	-0.800	-0.200
Guttiferae	0.600	0.429	0.200
Rosaceae	-0.300	0.300	,900*
Saliacea	0.500	-0.500	-0.500

Legend: *Correlation is statistically significant at p<0.05, **Correlation is statistically significant at p<0.01

EV-D68, is an emerging enterovirus, causing outbreaks, associated with mild and severe respiratory illness mainly affecting children (**Aliabadi *et al.*, 2016**; **Hu *et al.*, 2019**; **Mishra *et al.*, 2019**). No specific therapy is available neither to treat EV-D68 infection nor to prevent virus associated diseases (**Oermann *et al.*, 2015**). Therefore, natural products with potent antiviral activity could be considered as a source of novel therapeutic agents, helping to overcome this serious public health issue. Furthermore, no hive product has been assessed so far regarding its antiviral activity against EV-D68.
Although a direct comparison is not feasible, 7 BB and one BCP samples have demonstrated potent antiviral activity compared to other bee hive products. A recent study has shown that the well-known manuka honey exerted higher activity against the influenza virus compared to other honeys (**Watanabe *et al.*, 2014**) but still its SI value is lower than five of our samples against EV-D68.

CONCLUSION

This is the first study to test the antiviral properties of BCP and BB. We have shown that all BB and BCP samples exert significant antiviral activity against the tested virus (EV-D68) and in most cases viral inhibition is observed at concentrations that were non toxic for Rd cells. Although the correlation of antiviral potency to botanical origin is not totally conclusive, it has revealed that certain Brassicaceae and Rosaceae species correlate with higher antiviral activity. Further investigation will elucidate the antiviral compounds present in Greek BCP and BB and might lead to development of novel therapeutic agents.

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