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Variation in hemolymph content and properties among three Mediterranean bee species

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Abstract: Hemolymph, as mediator of immune responses and nutrient circulation, can be used as physiological marker of an insect's health, environmental quality or ecological adaptations. Recent studies reported intraspecific variation in protein contents and biological activities of the hemolymph of honey bees related to their diet. Here we measured interspecific variation in three common bee species in the Mediterranean Basin with contrasting ecologies: *Apis mellifera*, *Chalicodoma siculum*, and *Xylocopa pubescens*. Despite all the bees were collected in the same area, we found important quantitative and qualitative variations of hemolymph extracts across species. Samples of *A. mellifera* and *C. siculum* had much higher protein concentration, anticancer, antimicrobial and antioxidant activities than samples of *X. pubescens*. This first descriptive study suggests life history traits of bee species have strong influences on their hemolymph properties and call for future large scale comparative analyses across more species and geographical areas.

Keywords: Honey bees; carpenter bees; Megachilids; hemolymph; proteome; anticancer; antibacterial; antioxidant

1. Introduction

Bees are a large and diverse taxonomic group of about 20,000 species categorized in seven families [1]. Many of these species provide well-known natural products such as honey, bee pollen, wax, royal jelly and venom that have antioxidant, antibacterial or antitumor activities [2]. Increasing evidence suggest bee hemolymph also possesses interesting biological properties [3–6].

Hemolymph is a vital fluid involved in nutrient circulation to nourish tissues and in immune responses to fight infections [7]. Bee hemolymph contains various hydrophilic (e.g. hemocyanin) and hydrophobic (e.g. apolipoporphins) proteins [8–10]. High concentrations of these proteins improve the immune responses of bees and their resistance to infections [11–13]. Recent work described significant intraspecific variations in the proteomic profiles of the hemolymph of bees that are likely related to the diet [6,14]. Therefore, it has been proposed that the proteomic structure of hemolymph

38 can be used to monitor the physiological conditions of bees as well as the quality of their environment.
39 Since bees are found across a wide range of habitats worldwide [1], we also expect hemolymph
40 variation across species to exist and reflect key ecological adaptations.

41 Here we explored interspecific variations in hemolymph properties of bees from the
42 Mediterranean Basin. We analysed hemolymph samples of three common species collected in the
43 same area in Egypt: *Apis mellifera*, *Xylocopa pubescens* and *Chalicodoma siculum* (Figure 1). *A.*
44 *mellifera* (honey bees) are social Apidae nesting in cavities [15]. *X. pubescens* (carpenter bees) are
45 solitary Apidae that build their nests in wood [16,17]. *C. siculum* are solitary Megachilidae that build
46 their nests with mud [18]. This is a descriptive study that primarily aimed at analyzing honey bee
47 hemolymph and comparing it to that of two other common yet poorly studied bee species (*C. siculum*
48 and *X. pubescens*).

49

50 **2. Materials and Methods**

51 **Bees**

52 We sampled bees from 11 species (see details in Table 1) with a sweep net [19] between October
53 2020 (*A. mellifera*) and February 2021 (*X. pubescens* and *C. siculum*) in cultivated locations planted
54 with faba beans (*Vicia faba*) in the Ismailia Governorate area (Egypt). We collected all specimens from
55 each species in the same location on the same day. We then stored the bees at -20 °C until extraction
56 and analysis of the hemolymph [20]. Since we only obtained sufficient amounts of hemolymph for
57 three large-bodied species (*A. mellifera*, *X. pubescens*, *C. siculum*), we focused all our further
58 analyses on these three species (Table 1).

59

60 Table 1. Details about bee sampling and hemolymph extraction.

61

62 Hemolymph extraction and preparation

63 We extracted hemolymph from the specimen by using sterile insulin syringes to puncture the body
 64 close to the membrane of the coxa and applying a slight pressure on the abdominal region. We pooled
 65 the hemolymph samples from the same bee species and kept them in sterile Eppendorf tubes at -20
 66 °C until lyophilization by using freeze drying [20]. We then dissolved the weighed lyophilized
 67 hemolymph samples in two different but complementary solvents (a hydrophilic solvent: phosphate
 68 buffer saline – PBS [21]; and a hydrophobic solvent: dimethyl sulfoxide – DMSO [22]) in order to
 69 extract a maximum of molecules as the polarity of the solvent influences the protein structure,
 70 solubility and stability [23]. For each test both PBS and DMSO were used as negative controls.

71

72 Analysis of the protein content

73

74 Protein concentration

75 We measured the protein concentration (mg/mL) of the hemolymph extracts following Desjardins [24]
 76 using a Thermo Scientific™ (Waltham, MA, USA) Nano Drop™. One Micro volume UV-Vis
 77 Spectrophotometer with Bovine serum albumin (BSA) as standard in each sample (2 µL, 1 mg/mL).
 78 We analyzed three samples per bee species.

79

80 SDS-polyacrylamide gel electrophoresis

81 We used gel electrophoresis to separate protein bands of the hemolymph extracts based on their
 82 molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Bee species	Body size	Number of specimens	Weight of lyophilized hemolymph (grams)	Amount of hemolymph (microliter)	Location
<i>Apis mellifera</i>	Medium	81	0.15	~800	Ismailia
<i>Xylocopa pubescens</i>	Large	20	0.34	~1700	Ismailia
<i>Chalicodoma siculum</i>	Medium	29	0.106	~590	Ismailia
<i>Andrena savignyi</i>	Medium	50	<0.01	< 50	Ismailia
<i>Osmia latreillei</i>	Medium	<10	<0.001	< 50	Ismailia
<i>Colletes lacunatus</i>	Medium	16	0.039	< 50	Ismailia
<i>Amegilla quadrifasciata</i>	Medium	60	0.0029	< 50	Ismailia
<i>Megachile flavipes</i>	Medium	19	<0.001	< 50	Ismailia
<i>Thyreus bucinatus</i>	Large	6	<0.001	< 50	Ismailia

83 following Laemmli [25] with modifications. We mixed equal volumes of hemolymph samples with
84 solubilizing buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 25(w/v) SDS, 0.5% 2-mercaptoethanol
85 and 0.01% bromophenol blue). After heating for 4 min at 95°C, we inserted the samples into the wells
86 (15 µL sample with 100 µg/mL protein concentration per well) of the separating gel (12% PAGE gel).
87 We then ran an electrophoresis at constant 35 mA for 2 h using Consort N.V. (Belgium) mini vertical
88 electrophoresis system with running buffer. We prepared the staining gel using 0.1% Coomassie
89 Brilliant Blue (R-250) for the visualization of the protein bands.

90

91 *High performance liquid chromatography*

92 In complement to the electrophoresis, we performed an HPLC and analyzed the chromatograms by
93 investigating the protein peaks and their separation based on their retention time. We obtained equal
94 concentration (5 mg/mL) of hemolymph extracts in each of the two solvents (PBS or DMSO). We
95 analysed the extracts (70 µL) using a YL9100 HPLC System with Stationary phase C18 column
96 (Promosil C18 Column 5 µm, 150 mmx4.6mm) with acetonitrile (ACN) gradients of (10% - 100%)
97 acetonitrile in water mobile phase for 50 min at flow rate = 1 mL/min. The chromatogram was detected
98 using a UV detector at wavelength 280 nm following Basseri [26] with some modifications.

99

100 **Analysis of biological activities**

101

102 *Anticancer activity*

103 We measured antitumor activities of hemolymph extracts using a 3-[4,5-methylthiazol-2-yl]-2,5-
104 diphenyl-tetrazolium bromide (MTT) assay [27]. We seeded the cells from human liver cancer
105 (HepG2) and human cervical cancer (HeLa) in 96-well plate for 24 h (5x10³cells/well). After
106 incubation, we treated the cells with 100 µL of the hemolymph extracts at serial concentrations in PBS
107 and DMSO solvents (31.25, 62.5, 125, 250, 500 and 1000 µg/mL) and incubated them at 37 °C in 5%
108 CO₂ atmosphere for 48 h. We then washed the cells using PBS. We added fresh medium with MTT
109 dye and incubated at 37°C for 4 h. We then added DMSO for the solubilisation of the formazan
110 crystals in the viable cells [28]. We used a Bio-Tek ELISA micro plate reader to measure the
111 absorbance at 540 nm. The experiment was performed in triplicates. We calculated the percentage of
112 the cell viability (%) as follows:

$$113 \quad \text{Cell viability (\%)} = (A_T / A_C) \times 100.$$

114 Where A_T was the absorbance of treated cells with extracts, and A_C was the absorbance of the control
115 cells (untreated cells). The IC₅₀ values (i.e. concentration of extracts that cause inhibition in the growth
116 of 50% of the cells) were calculated for each sample using a dose-response curve with dose
117 concentration (X-axis) and cell cytotoxicity percentage (Y-axis).

118

119 *Antibacterial activity*

120 We analyzed the antimicrobial activity of the hemolymph using the agar well diffusion method of
121 Magaldi [29] against four bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*
122 and *Salmonella typhimurium*). We inoculated the agar plate surface by distributing bacterial

123 suspension. We then made a 6.0 mm hole aseptically using a sterilized tip and added 100 μ L of each
124 extract (10 mg/mL) or control into the well. We used PBS and DMSO as negative controls and
125 Gentamycin as a positive control. After the incubation, we expressed the in vitro antimicrobial activity
126 as inhibition zones in millimetres (mm) [30,31].

127

128 *Scavenging ability*

129 We measured the scavenging ability of hemolymph extracts using 1,1-Diphenyl-2-picrylhydrazyl
130 (DPPH) free radical [32]. We incubated a mixture of 100 μ L DPPH methanolic solution (0.004% in 95%
131 methanol) and 300 μ L of each hemolymph extracts at concentration 1 mg/mL and standard at 25 $^{\circ}$ C in
132 the dark for about 30-60 min. We used ascorbic acid (Vitamin C) as positive control, and measured
133 the colour changes using a spectrophotometer at 515 nm. We calculated the DPPH scavenging
134 activity of the samples using the following equation [33]:

$$135 \quad \text{Antioxidant activity (\%)} = 100 \times [(A_N - A_E)/A_N],$$

136 Where A_N was the absorbance of the negative control, and A_E was the absorbance of the sample or of
137 the standard.

138

139 *Hemolytic activity assay*

140 We assessed the hemolysis activities of the bee hemolymph extracts against human erythrocytes by
141 applying procedures of Malagoli [34]. We added blood samples to test tubes containing blood
142 anticoagulant (EDTA) and centrifuged the tubes for 5 min at 10,000 rpm. We then suspended red
143 blood cells in sterile PBS and incubated them with 100 μ L volume from series of various
144 concentrations of the tested extracts (range: 156.25 - 5000 μ g/mL). After incubation of the tubes at
145 room temperature for one hour, we centrifuged the tubes for 5 min at 1×10^3 rpm and measured the
146 absorbance of the supernatant at 570 nm. We used Triton 10% as positive control while PBS and
147 DMSO 10% as negative controls. We ran triplicate analysis for each bee species. We then calculated
148 the hemolysis percentage for each extract as follows:

$$149 \quad \text{Hemolysis (\%)} = 100 \times [(A_S - A_N) / (A_P - A_N)].$$

150 Where A_S was samples absorbance, A_N was the negative control absorbance, and A_P was the positive
151 control absorbance.

152

153 **Statistical analysis**

154 We analyzed the data in SPSS 22.0. We used Student's t-tests to compare protein concentrations and
155 IC_{50} of the hemolymph extracts in PBS and DMSO. We compared parameters of bee hemolymph (i.e
156 protein concentrations, IC_{50} s, antibacterial and antioxidant activities in either PBS or DMSO) across
157 species using one-way ANOVAs followed by Tukey's HSD post-hoc tests. We used two-way ANOVAs
158 to compare parameters of bee hemolymph (i.e. protein concentrations, IC_{50} s, antibacterial and
159 antioxidant activities) in both solvents among the three bee species. We considered significant
160 differences between samples when the p-value was lower than 0.05. All means are reported with their
161 standard error (mean \pm SE).

162 **3. Results**

163

164 Analysis of protein content

165

166 Protein concentration

167 Protein concentration of hemolymph varied across species and solvents (Two-ways ANOVA, species
168 x solvent: $F(2, 12) = 111.97, P < 0.001$; Figure 1). Highest protein concentrations were recorded for *C.*
169 *siculum* in PBS (0.27 ± 0.01 mg/mL, $n=3$) and DMSO (0.107 ± 0.01 mg/mL, $n=3$). Lowest protein
170 concentrations were recorded for *X. pubescens* in PBS (0.02 ± 0.005 mg/mL, $n=3$) and DMSO (0.06
171 ± 0.001 mg/mL, $n=3$). Hemolymph of *A. mellifera* had values falling in between for PBS and similar
172 values as *C. siculum* for DMSO.

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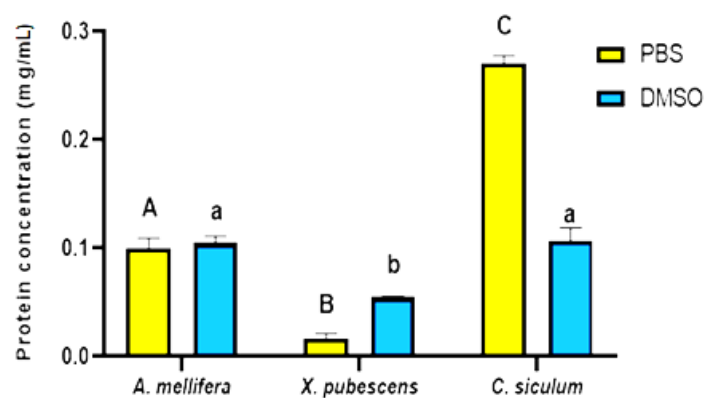
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184 Figure 1. Protein concentration in mg/mL for hemolymph extracts of three bee species (*Apis mellifera*,
185 *Xylocopa pubescens* and *Chalicodoma siculum*) in PBS or DMSO. Bars and error bars show the mean
186 \pm SE of triplicate results. Upper-case letters represent indicate significant differences between PBS
187 extracts and lower-case letters indicate significant differences between DMSO extracts (Tukey's HSD
188 test: $P \leq 0.05$).

189

190 SDS-polyacrylamide gel electrophoresis

191 Protein bands in electrophoresis gels of hemolymph extracted in PBS and DMSO had a molecular
192 weight ranging from ~5 to 250 kDa (Figure 2).

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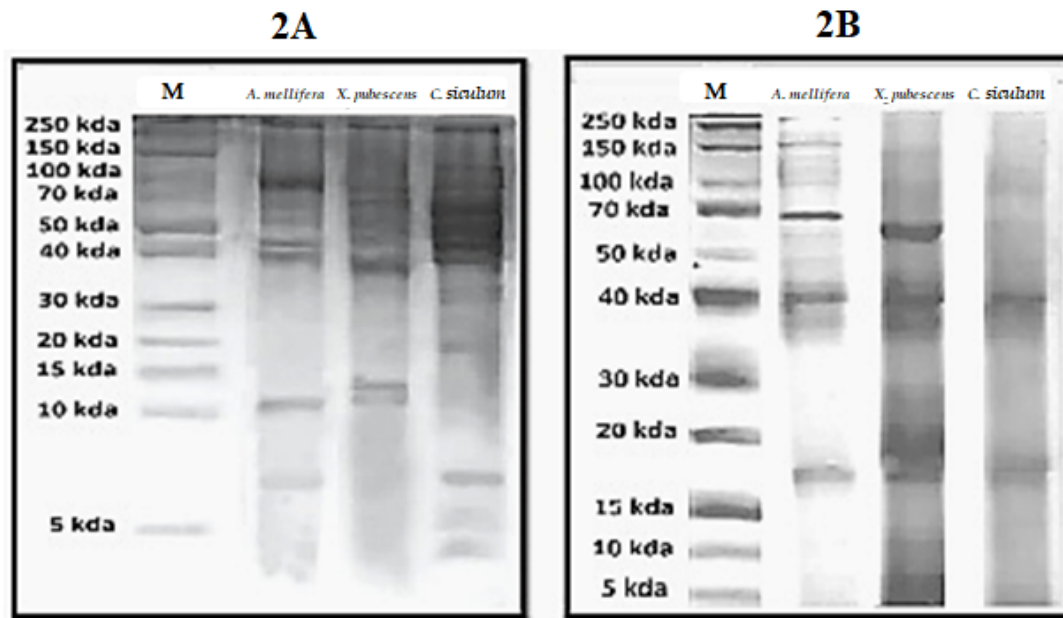
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The PBS dissolved extracts were characterized by five bands common to the three bee
species with molecular weights of ~40, ~60, ~70, ~110 and ~250 kDa (Figure 2A). Other bands
however were only observed in some species. A band with molecular weight of ~7 kDa was only
recorded in *A. mellifera* and *C. siculum*. Another band with molecular weight of ~10 kDa was only
recorded in *A. mellifera* and *X. pubescens*. Five bands with molecular weights of ~4, ~5, ~17, ~37 and
~100 kDa were exclusively recorded in *C. siculum*.

199 The DMSO dissolved extracts were characterized by five bands common to the three bee
 200 species with molecular weights of ~5, ~17, ~40, ~60 and ~110 kDa (Figure 2B). A band with molecular
 201 weight of ~150 was recorded in *A. mellifera* and *X. pubescens*. Two bands with molecular weights of
 202 ~10 and ~25 kDa were only recorded in *X. pubescens*.



203 Figure 2. Pictures of SDS-PAGE gel for hemolymph of the three bee species (*Apis mellifera*, *Xylocopa*
 204 *pubescens* and *Chalicodoma siculum*) extracted in (A) PBS or (B) DMSO. M is the marker.

205

206 RP-HPLC analysis

207 By analyzing the chromatogram of the PBS dissolved hemolymph extracts we found the presence of 6
 208 peaks common to the three bee species at retention times of 7.5, 11.1, 14.4, 15.4, 22.9 and 28.3 min
 209 (Table 2). In DMSO dissolved extracts, we detected 10 peaks common to the three bee species at
 210 retention times of 9.5, 23.3, 24.6, 25.5, 26.8, 28.3, 32.6, 33.2, 40.3 and 44.8 min (Table 2).

211

212 Table 2. HPLC most common chromatogram peaks profiles of the hemolymph of three bee species
 213 (*Apis mellifera*, *Xylocopa pubescens* and *Chalicodoma siculum*) extracted in PBS or DMSO. N.A.: not
 214 applicable.

215

Retention time (minutes)	Peak area (x10 ² mV.s)					
	<i>A. mellifera</i>		<i>X. pubescens</i>		<i>C. siculum</i>	
	PBS	DMSO	PBS	DMSO	PBS	DMSO
7.5	4.6	N.A	0.7	N.A	177.4	N.A

9.5	N.A	327	N.A	7.7	N.A	9.7
11.1	2.3	N.A	0.4	N.A	25.2	N.A
14.4	5.9	N.A	0.3	N.A	10.3	N.A
15.4	5.5	N.A	0.4	N.A	2.2	N.A
22.9	13.9	N.A	1.2	N.A	3.5	N.A
23.3	N.A	3.6	N.A	0.1	3.6	3.4
24.6	N.A	3.4	N.A	0.6	N.A	1.4
25.5	9.1	3.6	1.5	0.1	N.A	3.8
26.8	N.A	4.0	1.1	0.3	2.8	0.6
28.3	5.3	1.5	1.5	0.4	1.2	0.7
32.6	N.A	1.4	N.A	2.2	N.A	1.0
33.2	N.A	0.3	N.A	1.3	N.A	0.8
40.3	N.A	0.1	N.A	0.1	N.A	0.1
44.8	N.A	1.0	N.A	1.2	N.A	0.9

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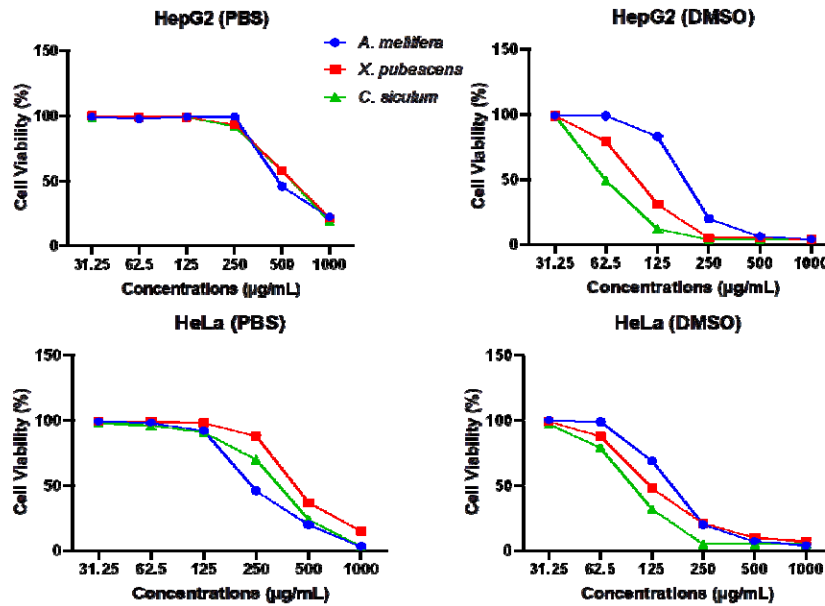
218 **Analysis of biological activities**

219

220 *Anticancer activity*

221 We evaluated the antiproliferative actions of the hemolymph extracts against the viability of hepatic
222 and cervical carcinoma cells. All hemolymph extracts resulted in inhibition of the cell viability against
223 the tested cancer cell lines in a dose-dependent manner after 48 h of incubation, irrespective of the
224 solvent used (Figure 3). However, the DMSO dissolved extracts showed higher overall cytotoxic
225 activity than the PBS dissolved extracts for HepG2 and for HeLa (Two-ways ANOVA, HepG2: $F(2, 12) = 26.667$, $P < 0.001$; HeLa: $F(2, 12) = 117.111$, $P < 0.001$) (Figure 4).
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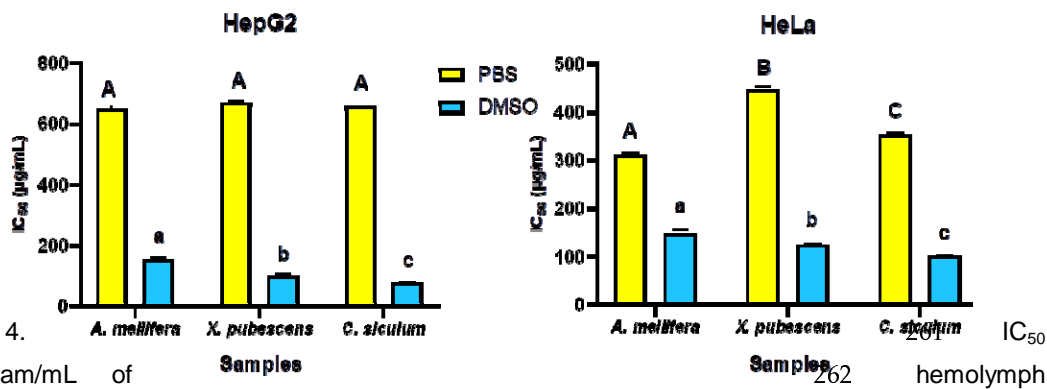


243 Figure 3. Effects of hemolymph extracts of three bee species (*Apis mellifera*, *Xylocopa pubescens* and
244 *Chalicodoma siculum*) on cell proliferation of human cancer (HepG2 and HeLa) cell lines at different
245 concentrations in PBS or DMSO.

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Among the DMSO dissolved extracts, *C. siculum* hemolymph had the highest cytotoxic effects against
HepG2 and HeLa cell lines (IC₅₀ HepG2 = 77.35 µg/mL, IC₅₀ HeLa = 101.2 µg/mL). The lowest
cytotoxic effects were recorded for *A. mellifera* hemolymph (IC₅₀ HepG2 = 153.1 µg/mL, IC₅₀ HeLa =
148.46 µg/mL) (Figure 4). Among the PBS dissolved extracts, *A. mellifera* hemolymph had the highest
cytotoxic effects (IC₅₀ HepG2 = 649.4 µg/mL, IC₅₀ HeLa = 312.54 µg/mL) and *X. pubescens* had the
lowest (IC₅₀ HepG2 = 669.2 µg/mL, IC₅₀ HeLa = 447.2 µg/mL) (Figure 4).

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263 Figure 4. IC₅₀ in µgram/mL of
264 hemolymph extracts of three bee species (*Apis mellifera*, *Xylocopa pubescens* and *Chalicodoma siculum*) against
265 HepG2 and HeLa cell lines, using PBS or DMSO as solvents. Bars and error bars represent the mean
values ±SE of triplicate results. Upper-case letters indicate significant difference between PBS extracts

266 and lower-case letters indicate significant differences between DMSO extracts (Tukey's HSD test: $P \leq$
 267 0.05).

268

269 *Antimicrobial activity*

270 We observed the antibacterial activity of hemolymph extracts against Gram-positive and Gram-
 271 negative bacteria (Table 3, see example in Figure 5). The results showed comparable antibacterial
 272 activities of hemolymph extracts among the tested bee species and solvent; *Bacillus subtilis* (ANOVA,
 273 $F(2, 12) = 0.800, P=0.472$), *Staphylococcus aureus* (ANOVA, $F(2, 12) = 0.042, P=0.960$),
 274 *Escherichia coli* (ANOVA, $F(2, 12) = 2.054, P=0.171$) and *Salmonella typhimurium* (ANOVA, $F(2, 12)$
 275 $= 2.771, P=0.102$).

276

277 Table 3. Inhibition zone (mm) of hemolymph from three bee species (*Apis mellifera*, *Xylocopa*
 278 *pubescens* and *Chalicodoma siculum*) extracted in PBS or DMSO against various types of bacteria
 279 (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*). CON:
 280 Gentamycin as positive control, N.A: not applicable, \pm S.E.: standard error values obtained from
 281 triplicate measurements. Values with different superscript letters in a column are significantly different
 282 (Tukey's HSD test: $P \leq 0.05$), a versus *A. mellifera*, b versus *X. pubescens* and c versus *C. siculum*.

Sample Pathogen	PBS			DMSO			CON.
	<i>A. mellifera</i>	<i>X. pubescens</i>	<i>C. siculum</i>	<i>A. mellifera</i>	<i>X. pubescens</i>	<i>C. siculum</i>	
<i>B. subtilis</i>	34 \pm 1.0 ^{bc}	26 \pm 0.3 ^a	26 \pm 0.8 ^a	32 \pm 1.6 ^b	25 \pm 0.8 ^a	27 \pm 1.4	22 \pm 0.6
<i>S. aureus</i>	25 \pm 2.0	25 \pm 0.8	23 \pm 1.4	24 \pm 0.9	25 \pm 0.3	22 \pm 0.5	15 \pm 0.9
<i>E. coli</i>	40 \pm 1.5 ^{bc}	24 \pm 1.4 ^a	25 \pm 0.5 ^a	38 \pm 1.0 ^{bc}	22 \pm 0.8 ^a	23 \pm 0.3 ^a	17 \pm 1.0
<i>Salmonella typhimurium</i>	N.A	25 \pm 1.1	24 \pm 1.1	N.A	21 \pm 0.8	22 \pm 1.1	23 \pm 0.7

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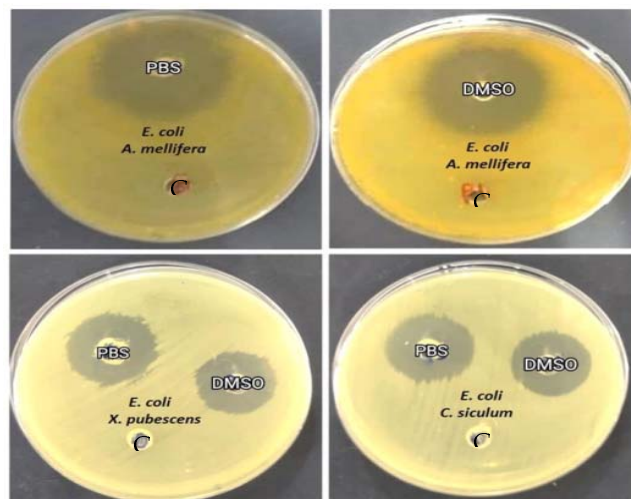
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295 Figure 5. Antimicrobial activity of hemolymph from three bee species (*Apis mellifera*, *Xylocopa*
296 *pubescens* and *Chalicodoma siculum*) extracted in PBS or DMSO against *E. coli* bacteria and by
297 using negative control.

298

299 *DPPH radical scavenging assay*

300 The hemolymph extracts of *A. mellifera*, *X. pubescens* and *C. siculum* possessed effective scavenging
301 actions in both PBS and DMSO (Figure 6). Overall, the hemolymphs of the different species showed
302 different antioxidant activities (two-ways ANOVA, species: $F(2, 12) = 5.06$, $P=0.025$) and these values
303 were higher in PBS dissolved extracts (two-ways ANOVA, solvents: $F(1, 12) = 10.729$, $P=0.007$).
304 However, for a given species the antioxidant activity in both solvents was comparable (two-ways
305 ANOVA, species x solvent: $F(2, 12) = 2.451$, $P=0.128$).

306 The highest antioxidant activity was reported for *A. mellifera* PBS extracts (29.1%) and *C.*
307 *siculum* DMSO extracts (25.2%), while *X. pubescens* reported the lowest antioxidant activity (25.7%)
308 in PBS and (19.3%) in DMSO (Figure 6).

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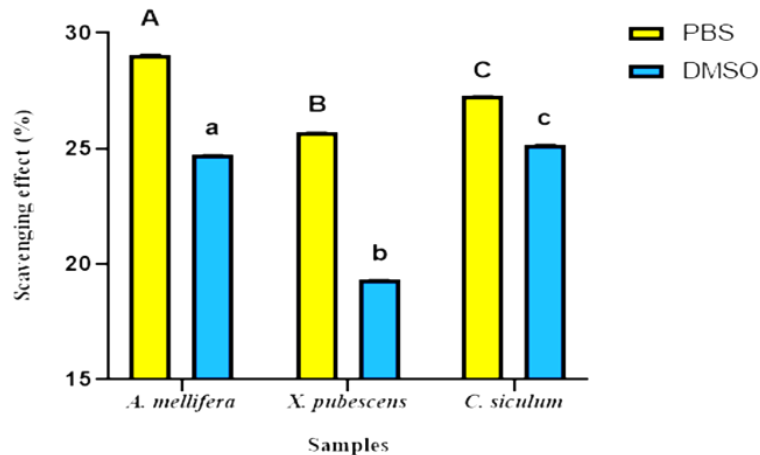
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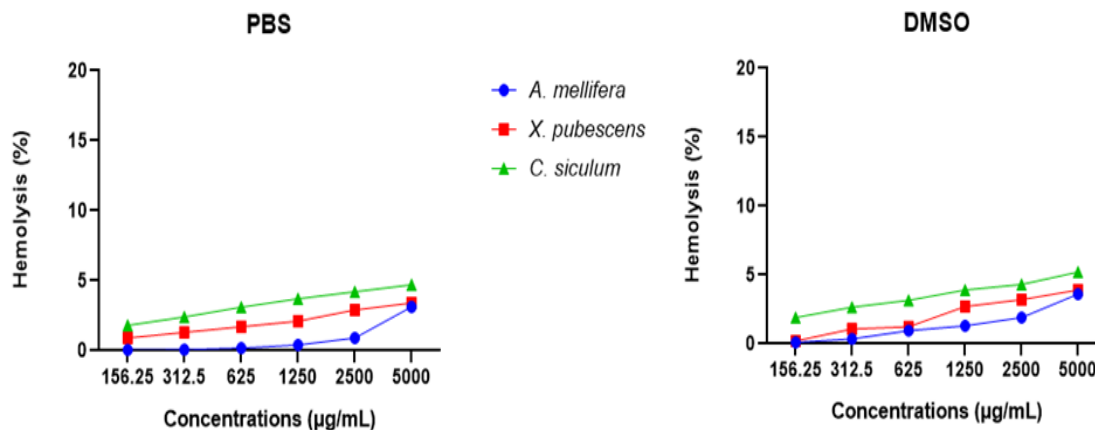
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329 *Hemolytic activity*

330 Hemolytic properties of the bee hemolymphs were tested against human erythrocytes. For the three
331 bee species, hemolymph extracts possessed either no or low hemolytic activity against human
332 erythrocytes in reference to negative (PBS and DMSO 10%) and positive controls (Triton10%) (Figure
333 7).

334



335 Figure 7. Hemolytic activity of hemolymph extracts from three bee species (*Apis mellifera*, *Xylocopa*
 336 *pubescens* and *Chalicodoma siculum*) at different concentrations against human erythrocytes.
 337 Hemolymph samples were extracted in PBS or DMSO.
 338

339 4. Discussion

340 We aimed to evaluate the interspecific variability in hemolymph protein content and biological activities
 341 from bees collected in the same locality in Northern Egypt. We found considerable variation among
 342 the three bee species for which we managed to extract enough hemolymph after our sampling
 343 campaign (see summary results in Table 4).
 344

345 Table 4. Summary of analysis of hemolymph of three bee species (*Apis mellifera*, *Xylocopa*
 346 *pubescens* and *Chalicodoma siculum*) extracted in two solvents (PBS and DMSO). (+): high, (+/-):
 347 medium and (-): low.
 348

	<i>A. mellifera</i>		<i>X. pubescens</i>		<i>C. siculum</i>	
	PBS	DMSO	PBS	DMSO	PBS	DMSO
Protein concentration	(+/-)	(+/-)	(-)	(-)	(+)	(+/-)
Anticancer activity	(+)	(-)	(-)	(+/-)	(+/-)	(+)
Antimicrobial activity	(+)	(+)	(+/-)	(+/-)	(+/-)	(+/-)
Antioxidant activity	(+)	(+/-)	(-)	(-)	(+/-)	(+)
Hemolytic activity	(-)	(-)	(-)	(-)	(-)	(-)

349

350 Overall, *X. pubescens* hemolymph possessed the lowest protein concentrations, *A. mellifera*
351 hemolymph had intermediate values, and *C. siculum* hemolymph showed highest protein
352 concentrations irrespective of the solvent used for extraction. Previous studies reported intraspecific
353 variations in hemolymph protein concentration primarily correlated to the diet of bees, even among
354 bees from the same worker caste [6,35–37]. Since all our samples came from the same study site and
355 were collected on the same plants, this suggests *A. mellifera*, *X. pubescens* and *C. siculum* had
356 different diets. Bee diet varies based on various parameters [38] including the physiological states of
357 the bees themselves and the developmental stages of larvae they need to feed [39–41]. Another
358 source of variation is the impact of presence of phoretic mites on *Xylocopa* species that feeds on
359 pollen paste [42], the main source of proteins in the hemolymph of bees [43].

360 The lowest protein concentrations in *X. pubescens* were associated with the weakest
361 antioxidant and antibacterial activities against both *B. subtilis* and *E. coli*. This was also associated
362 with the weakest anti-proliferative activities among the PBS dissolved hemolymph extracts against
363 both HepG2 and HeLa cell lines. By contrast, the highest protein concentrations of *C. siculum* in both
364 solvents were associated with the strongest anti-proliferative activities among DMSO dissolved
365 hemolymph extracts against both tested cancer cell lines. This is consistent with the fact that high
366 protein concentration in the bee hemolymph is associated to high resistance to pathogens, increased
367 life span, and improved immunity [11–13]. Therefore protein concentration in hemolymph is a good
368 indicator of bee health.

369 SDS-PAGE gel analysis revealed proteomic profiling of the hemolymph extracts of the three
370 bee species in PBS or DMSO solvents. Protein bands common to the three species, with molecular
371 weight (4-5 kDa) in DMSO dissolved hemolymph were similar to cercopin, a family of small proteins
372 have been isolated from different insect hemolymph which possesses anti-bacterial properties against
373 both Gram positive and Gram negative bacteria [44,45]. Extractions in DMSO also revealed another
374 protein bands common to the three species that were detected at molecular weight (≈17 kDa) similar
375 to a group of lectins [46]. Lectin is a defense protein that can participate in many biological activities
376 such as antimicrobial, antioxidant and anticancer in arthropods. It can be also considered as natural
377 anticancer agent and contribute in immune actions [47–53]. CLIPC9 similar bands were observed at
378 (≈37 kDa) in both PBS and DMSO extracts consistent with their detection in the hemolymph of
379 *Anopheles gambiae* [54]. CLIP proteases are serine proteases found in the hemolymph of all insects
380 and participate in the innate immune responses. The protein bands with molecular weight (≈40 kDa)
381 were also found in both PBS and DMSO extracts are in the same range of lipopolysaccharide
382 recognition protein (LRP). LRP was purified from the plasma of large beetle larvae, *Holotrichia*
383 *diomphalia* with immune roles as it participates in agglutinating activities against *E. coli* and other
384 bacteria [55]. Protein bands detected at molecular weight (≈60 kDa) in PBS or DMSO extracts are
385 within the range of purified protein fractions extracted from cockroach hemolymph with potent
386 antimicrobial activities [26]. Moreover, common detected protein bands at (≈70 kDa) were similar to
387 hemocyanin, an oxygen transporter protein that possesses antioxidant, antiparasitic, antimicrobial,
388 anticancer and other biotic activities. This protein is found in most arthropods [53,56,57].

389 The variety and likely dominance of the bioactive proteins such as cercopins, lectins, CLIP
390 proteases, LRP and hemocyanin in PBS and DMSO hemolymph extracts may clarify the effective
391 biological activities of hemolymph in the current study. Generally, DMSO dissolved hemolymph
392 extracts possessed higher cytotoxic activities against HepG2 and HeLa cancer cell lines than the
393 effect of PBS extracts. Our proteomic profiling analysis revealed the presence of variations according
394 to the type of dissolved proteins in different solvents, in agreement with studies on the impact of
395 solvent on the protein structure [21,58]. According to Kramer [59] and Nugraha [21], altered solvents
396 affects the protein solubility thus may influences the hemolymph bio-activities. Hydrophobic proteins
397 such as lipoprotein complexes were used as delivery vehicles for anticancer drugs [60].

398 The fact that none of the hemolymph extracts possessed lytic activities against erythrocytes
399 agrees with results of previous study on the hemolytic activities of honey bee hemolymph [6]. These
400 results may encourage the evaluation of bee hemolymph extracts as safe and selective therapeutic
401 agents. Accordingly, bee hemolymph mixed with herbal extracts exhibited high anticancer efficiency
402 with less or no hemolytic activities [4].

403 Honey bees have innate immune mechanisms including the physical barriers and both
404 humoral and cellular actions for their defense against infections and pathogens that affects the bee
405 immune system and thus affects the bee health and the social behavior of these insects develops their
406 social immunity which reduces the stress of the individual immune response of the bees [61]. Further
407 studies are required on the immune responses of other different bee species for explaining the
408 variations in their proteomic content and biological activities

409 **5. Conclusions**

410 Our study highlights significant interspecific variability in the protein concentration and biological
411 activities of the hemolymph of three common Mediterranean bee species, likely related to variation in
412 their life history traits. Broader scale comparative studies, using similar procedures as ours, across
413 different geographical areas are now needed to investigate variations among bee species and how
414 they are related to their ecology and evolution.

415

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417 Methodology, Software, validation, formal analysis, investigation, resources and data curation. **SE,**
418 **MT, IB, MS and ML:** Writing—Original Draft Preparation, Writing—Review & Editing, visualization. All
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426

427

428 **References**

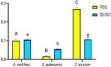
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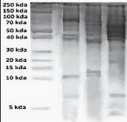
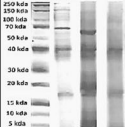
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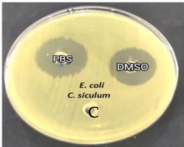
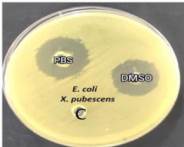
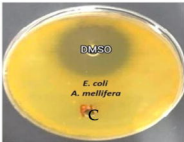
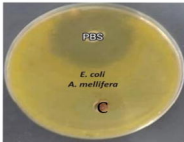
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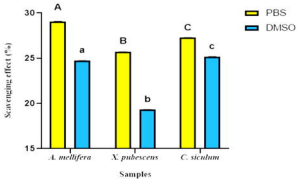
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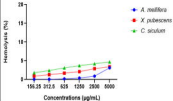
1A**M***A. nidulans* *S. pombe* *C. albicans***2B****M***A. nidulans* *S. pombe* *C. albicans*







PBS



DMSO

