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**A comparison of the chemical composition and biological activity of mature and immature honeys: an HPLC/QTOF/MS-based metabolomics approach**

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1 **Abstract:** Harvesting uncapped immature honey (IMH) followed by dehydration is a  
2 typical counterfeit honey production process, but the differences between IMH and  
3 capped mature honey (MH) have previously not been well described. In this study, MH  
4 and IMH from the *Apis mellifera* colonies in the same rapeseed flower season were  
5 compared. MH was found to have lower water content, acidity and higher fructose  
6 content. HPLC-Q-TOF/MS based untargeted metabolomic analysis indicated that MH  
7 had a distinct metabolite composition to IMH. Targeted metabolomic analysis on 20  
8 major polyphenolic constituents showed higher accumulation in MH. MH had greater  
9 bacteriostatic effect and stronger free radical scavenging effect. Whilst both honeys  
10 mitigated cell damage caused by H<sub>2</sub>O<sub>2</sub>, the effective dosage of IMH was higher and its  
11 inducing effect on the anti-oxidant gene expression was weaker. Overall, MH was  
12 shown to be of better quality than IMH not only because of its richer polyphenolic  
13 composition, but also due to its stronger biological activity.

14

15 **Keywords:** honey, mature, immature, metabolomic analysis, bioactivity, HPLC-Q-  
16 TOF/MS.

17

## 18 **Introduction**

19 Honey is a miraculous product resulting from millions of years of coevolution  
20 between plants and honey bees, *Apis* species.<sup>1</sup> It is a natural sweetener that originates  
21 from the plant nectar or honey dew collected by bees and further matured inside the bee  
22 hive.<sup>2</sup> Bees build a band of honeycomb above the brood cells in their nests to store  
23 honey and pollen. The mature honey is capped with white wax for long-term storage.<sup>2</sup>  
24 Stored honey and pollens act as food sources, whilst the honeycomb band provides  
25 insulation during the winter period or on days without foraging activities.<sup>3</sup>

26 The process of honey maturation begins with the forager bees taking the nectar or  
27 honey dew to the hives.<sup>2</sup> The forager transfers these carbohydrates from their stomach  
28 to storer bees.<sup>4</sup> Storer bees normally add their own substances, like enzymes from the  
29 hypopharyngeal glands to convert the sucrose into glucose or fructose.<sup>5</sup> The acids from  
30 the bees' stomach lowers the pH of the IMH. At the same time, the drying process by  
31 their evaporation behavior further decreases the moisture of the honey.<sup>2</sup> The duration  
32 of honey maturation varies from one to eleven days depending on-colony size, humidity,  
33 climatic conditions and the botanical origins of the nectar.<sup>6</sup> After the honey matures,  
34 bees cover the honey with a wax lid as protection and to prevent unwanted fermentation  
35 and spoilage.<sup>7</sup>

36 Due to its great value, honey has been subjected to fraud threat since ancient times.  
37 Counterfeit honeys remain a serious threat to the global beekeeping business. Typical  
38 frauds may involve diluting honey using a variety of syrups,<sup>8,9</sup> lightening honey color  
39 using ion-exchange resins,<sup>4</sup> labeling the honey with fraudulent geographical and/or

40 botanical origins,<sup>10</sup> artificial feeding of bees during a nectar flow and harvesting the  
41 immature (uncapped) honey.<sup>11</sup> The latter fraud type is quite prevalent, since some  
42 beekeepers think this can increase the honey harvest. The unmaturred honey then  
43 undergoes dehydration with vacuum dryers, resulting in most physiochemical features  
44 still falling within regulatory.<sup>11</sup> Collecting uncapped honey followed manual  
45 dehydration is now regarded as an illicit practice. It is already accepted that water  
46 content might be a major difference between mature (capped) and immature (uncapped)  
47 honey.<sup>11</sup> However, during the natural transformation of nectar into honey, bees can add  
48 specific substances. The chemical composition of honey is complex, not only consisting  
49 of sugars and water, but also other constituents, including amino acids, vitamins,  
50 minerals and plant polyphenolic acids.<sup>12</sup> These components together endow honey with  
51 distinct flavors and biological activities.<sup>13</sup> Nevertheless, it remains to be determined  
52 whether these minor substances result in significant differences in chemical  
53 compositions and biological activities between mature honey (MH) and immature  
54 honey (IMH). To understand these two types of honey better, this study compared the  
55 chemical composition and biological activities (anti-oxidative and anti-microbial) of  
56 MH and IMH.

## 57 **Materials and methods**

### 58 *Chemicals and reagents*

59 Methanol (MeOH) and formic acid (FA) were purchased from Fisher Scientific  
60 Inc (Pittsburgh, PA, USA). Trolox, ascorbic acid, quercetin, gallic acid and other  
61 standards were purchased from Sangon biological engineering co. LTD (Shanghai,

62 China). Solid-phase extraction (C18) was purchased from Waters scientific Inc. LB  
63 Nutrient Agar was purchased from Beijing Aoboxing biotechnology co. LTD.  
64 *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* were obtained from  
65 Institute of microbiology, Chinese Academy of Sciences, China. The solid phase  
66 extraction (SPE) cartridges were obtained from Waters (Milford, Mass, USA). All the  
67 cartridges contained 500 mg of C<sub>18</sub>.

#### 68 *Honey sample collection and physicochemical analysis*

69 Raw honey samples were collected from three *A. mellifera* L. colonies in Sichuan,  
70 China, during the flower season from March 1<sup>st</sup> to March 30<sup>th</sup>, 2019. Three colonies  
71 with the same potential were selected from the experimental bee hive. The honey in the  
72 colonies was cleared and only a small amount was left for bees to maintain a basic life.  
73 The honey collected by bees and brought back to the nest for no more than 24 h was  
74 recorded as immature (uncapped) honey (IMH), and the honey stored in the honeycomb  
75 with a beeswax seal until the sealed area of beeswax was greater than 70% was recorded  
76 as capped mature (capped) honey (MH). Three IMH and MH samples were separately  
77 collected from each colony. A total of 18 samples were collected, including 9 MH and  
78 9 IMH, and stored at -20 °C in the dark prior to use.

79 These 18-batches of rapeseed honey were subjected to chemical analysis.  
80 Indicators including water, glucose, fructose, sucrose, acidity and 5-  
81 hydroxymethylfurfural (HMF) were determined as previously described.<sup>14</sup>

#### 82 *Preparation of active substances*

83 Five grams of honey sample was added into 10 mL deionized water followed by

84 sonicating—at 60 kHz for 10 min and centrifugation at 8000 r/min for 5 min. The  
85 supernatant was collected and added to the SPE cartridges that were preconditioned  
86 initially with 5 mL of methanol (MeOH) and then 5mL of water. The supernatant  
87 samples passed through the cartridges at a flow rate of approximately 1 mL/min. The  
88 analytes were eluted with 8 mL of methanol. The resulting eluate was dried using a  
89 nitrogen stream to obtain immature honey extract (IMHE) and mature honey extract  
90 (MHE). Both extracts were stored at -20 °C.

#### 91 *HPLC-Q-TOF/MS analysis of honey extract*

92 The honey extracts were re-dissolved to a pre-determined concentration with  
93 MeOH. The solution was then filtered with a 0.22 µm nylon membrane and placed in a  
94 brown vial. High performance liquid chromatography combined with quadrupole time-  
95 of-flight mass spectrometry (HPLC-Q-TOF/MS, 6545) system was used to perform the  
96 chromatographic analysis in the negative ionization mode. An Agilent Zorbax Poroshell  
97 EC-C18 column (2.1 mm x 100 mm, 2.7 µm) was used to separate the extracted  
98 compounds. Analytes were separated by linear gradient elution with ultrapure water  
99 containing 0.1% formic acid (v/v) (A) and MeOH (B) at a flow rate of 0.25 mL min<sup>-1</sup>.  
100 The linear gradient elution program was: 0–1 min, 5% B; 1–6 min, 55% B; 6–20 min,  
101 95% B; 20–26 min, 95% B; 26–27 min, 5% B. The column temperature was set to 30 °C  
102 with an injection volume of 2 µL. The parameters of ESI source were as follows: a  
103 nebulizer pressure of 40 psi, a capillary voltage of 3500 V, a fragmentor voltage of 120  
104 V, a drying gas (N<sub>2</sub>) flow rate of 8 L/min, a drying-gas temperature of 320 °C and a  
105 mass range of m/z 100–1700.

106 *Determination of total phenolic and flavonoid content*

107 The measurement of total polyphenol content in the honey extracts was determined  
108 by the Folinol- Ciocalteu method. 100  $\mu\text{L}$  of the extract was mixed with 100  $\mu\text{L}$  of Folin  
109 and Ciocalteu's phenol reagent. The mixture was incubated in the dark for 5 min,  
110 followed by the addition of 300  $\mu\text{L}$  sodium carbonate solution (2% w/v) and mixed.  
111 The reaction proceeded in the dark for 120 min. The absorbance was measured at 765  
112 nm. Gallic acid was used to calculate the standard curve and the results were expressed  
113 as mg of gallic acid equivalents (GAEs) per g of honey extraction.

114 For the measurement of total flavonoid content, 150  $\mu\text{L}$  of the sample was mixed  
115 with 10  $\mu\text{L}$  aluminium nitrate (100 g/L), 10  $\mu\text{L}$  potassium acetate (9.8 g/L) and 330  $\mu\text{L}$   
116 of distilled water. The reaction proceeded in the dark for 120 min. The absorbance of  
117 the product was determined at 415 nm. Quercetin was used to calculate the standard  
118 curve and the results were expressed as mg of Quercetin equivalents (QEs) per g of  
119 honey extraction.

120 *Antioxidant activity*

121 Free radical scavenging ability

122 Various concentrations of honey phenolic extracts (0.2 mL) were mixed with 0.2  
123 mL of ethanolic solution containing DPPH radicals. The mixture was shaken vigorously  
124 and left to stand for 30 min in the dark or until stable absorption values were obtained.  
125 The reduction of the DPPH radicals was determined by measuring the absorption at 517  
126 nm. The concentration of the extract providing 50% of radical scavenging activity  
127 (IC<sub>50</sub>) was determined by a linear curve established by mass concentration and



128 clearance. The results were expressed as mg of Trolox per g of honey extraction.  
129 Vitamin C was used as the positive control.

130 Various concentrations of honey phenolic extracts (0.15 mL) were mixed with 0.25  
131 mL of ethanol solution containing ABTS<sup>+</sup> working liquid. The mixture was shaken  
132 vigorously and left to stand for 10 min in the dark until stable absorption values were  
133 obtained. The reduction of the ABTS<sup>+</sup> radical was determined by measuring the  
134 absorption at 734 nm. The concentration of the extract providing 50% of radicals  
135 scavenging activity (IC<sub>50</sub>) was determined by a linear curve established by mass  
136 concentration and clearance. The results were expressed as mg of Trolox per g of honey  
137 extraction. Vitamin C was used as positive control.

#### 138 Reducing ability

139 Various concentrations of the honey extracts (0.3 mg) were mixed evenly with 75  
140 μL of sodium phosphate buffer (pH 6.6) and 75 μL of 1% potassium ferricyanide (w/v).  
141 The mixture was then incubated at 50 °C for 20 min. After 75 μL of 10% (v/v)  
142 trichloroacetic acid was added, the mixture was centrifuged at 2000 rpm for 10 min.  
143 The upper layer (300 μL) was mixed with 300 μL of deionized water and 60 μL of 0.1%  
144 of ferric chloride (v/v). Then the mixture was shaken, and the absorbance was measured  
145 spectrophotometrically at 700 nm. The concentration of the extract providing an  
146 absorbance of 0.5 (IC<sub>50</sub>) was determined by a linear curve established by mass  
147 concentration and absorbance. The results were expressed as mg of Trolox per g of  
148 honey extraction. Vitamin C was used as the positive control.

#### 149 *Cell culture and cell viability assay*

150 Mouse skin fibrocytes L929 cells were incubated in high-glucose Dulbecco's  
151 modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal  
152 bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin at 37 °C in an  
153 incubator with 5% CO<sub>2</sub>. Cells were then passaged once every 1.5 days. The toxicity of  
154 the honey extract and H<sub>2</sub>O<sub>2</sub> was determined by using a CCK-8 kit (Dojindo, Japan)  
155 following the manufacturer's instructions. The absorbance was measured at 450 nm  
156 using a microplate reader (Bio-Rad Model 550, CA, USA).

#### 157 *Total RNA isolation and quantification*

158 L929 cells were pretreated with designated concentrations of the honey extract for  
159 2 h, then stimulated with 500 µM H<sub>2</sub>O<sub>2</sub> for 24 h. Total RNA was collected and extracted  
160 using the RNA Pure Kit (Carry Helix Biotechnologies Co., Ltd., Beijing, China). The  
161 concentration and purity of the RNA measured using the Nano Drop 2000 ultramicro  
162 spectrophotometer. RNA was reverse transcribed by PrimeScript™ RT Master MIX  
163 kit (TaKaRa, Dalian, China) and the product stored at -20 °C.

164 Quantitative real-time PCR was implemented using Bioer LineGene 9600 system  
165 (Hangzhou, China) with the SYBR premix EXTaq (TaKaRa, Dalian, China) according  
166 to the two-step reaction method. The gene-specific primers of selected cytokines were  
167 listed in Supplemental Table 1. The expression of housekeeping gene GAPDH was used  
168 to normalize the expression levels of these target genes, the specificity was confirmed  
169 by dissociation curve analysis and gel electrophoresis. And the relative expression  
170 levels of target genes were calculated using  $2^{-\Delta\Delta C_t}$  method.

#### 171 *Anti-microbial activity*

172 Anti-microbial activity was measured by an agar diffusion method. LB agar was  
173 sterilized and cooled to 60 °C and 100 µL bacterial solution (10<sup>6</sup> CFU/mL) was added  
174 to each 30 mL agar to prepare the bacteria-containing medium. After the plate was set,  
175 the sample solution to be tested was evenly added into a sterilized Oxford cup (100  
176 µL/cup). The negative control was deionized water, and the positive control was  
177 ampicillin solution (5 g/mL). Plates were incubated at 37 °C for 16 h. A Vernier caliper  
178 was used to measure the diameter of the zone inhibition (in mm), and the average values  
179 were obtained by repeating the test in triplicate. The results were presented as a mean  
180 ± SD.

#### 181 *Statistical analyses*

##### 182 General analysis

183 Data was obtained from at least three independent experiments and shown as the  
184 mean ± SD of the indicated replicates. Statistical differences were analyzed using One  
185 way ANOVA test followed by Bonferroni post hoc analysis and Student's unpaired *t*-  
186 test  $P < 0.05$  was accepted as statistically significant.

##### 187 Untargeted metabolomics statistical analysis

188 Raw data obtained by HPLC-Q-TOF/MS system was preliminarily processed to  
189 provide structured data in an appropriate format for subsequent data analysis. The  
190 resulting data was extracted by the Profinder software tool in the MassHunter Qualitative  
191 Analysis Software (Agilent Technologies) and converted into CEF files. The list of all  
192 possible components, as represented by the full TOF mass spectral data, was created in  
193 this way. Each compound was described by mass retention, time, and abundance. Then

194 data filtering was performed with Mass Profiler Professional (Agilent Technologies)  
195 software. Before statistical analysis, filtration of data matrix by sample frequency was  
196 also applied. Only substances with a frequency greater than 70% were selected for  
197 further analysis. The sample differences were statistically analyzed by using One way  
198 ANOVA test followed by Bonferroni post hoc analysis and Student's paired  $t$ -test (again,  
199  $P < 0.05$  was considered significant).

200 The materials showing significant difference between groups were matched and  
201 analyzed by using Traditional Chinese Medicines (TCM) database (Agilent  
202 Technologies). Principal-component analysis (PCA) was also used to analyze the  
203 difference between samples, and score plots were produced.

204 Targeted metabolomics statistical analysis

205 Targeted compound ion chromatogram was extracted by Mass Hunter Qualitative  
206 Analysis software (Agilent Technologies) for all samples. We conducted qualitative  
207 analysis according to the retention time, molecular weight and mass spectrometry  
208 fragment and quantitative analysis through the external standard method. The peak  
209 areas were used to construct standard curves with  $R^2 \geq 0.99$ . A  $t$ -test of the quantitative  
210 results was performed to analyze the difference of phenolic substances in MH and IMH  
211 samples.

## 212 **Results**

### 213 *Physicochemical analysis*

214 To study the difference between mature (capped) honey (MH) and immature  
215 (uncapped) honey (IMH), physical and chemical indicators were assessed as shown in

216 Table 1. The indexes of MH were as follows: moisture content  $18.31 \pm 1.52\%$ , acidity  
217  $13.67 \pm 1.88$  mL/kg, total sugar content  $73.22 \pm 2.71\%$ , fructose content  $36.40 \pm 0.37\%$ .  
218 IMH: moisture content  $31.20 \pm 1.81\%$ , acidity  $19.9 \pm 0.42$  mL/kg, total sugar content  
219  $61.11 \pm 2.09\%$ , fructose content  $30.86 \pm 0.64\%$ . Compared with IMH, MH samples had  
220 lower water content, lower acidity and a higher fructose content.

## 221 *Metabolomic profiling*

### 222 Untargeted study

223 We enriched the active components in the honey and analyzed their differences in  
224 the honey extracts by metabolomics using Agilent MPP software. In the first step, the  
225 molecular features (MFs) that were present in all injections were retained for each  
226 species. The total number of the molecules were 3,751 from all injections, and  
227 significantly reduced to 3,060 after the filtering step. The results from the data analysis  
228 are represented by a Venn diagram (Figure. 1. A). The results showed that 2,572  
229 chemicals were detected in MH, and 2,686 substances were detected in IMH, with 2,198  
230 substances in common. Secondly, molecular features were further filtered based on p-  
231 values calculated by one-way ANOVA. A p-value cutoff of 0.05 was set as the filtering  
232 standard to maintain the MFs which differed significantly. The final filtering step was  
233 conducted using fold change (FC) analysis (Figure. 1. B). The value of FC was  
234 calculated as the MF abundance ratios between each of the two groups. Only the MFs  
235 with FC of 2.0 or higher abundance were picked out. As shown in Figure. 1. B, each  
236 grey dot represents a chemical while the red dots highlight those substances that were  
237 significantly up-regulated in MH group compared with those in IMH group. Equally,

238 the blue dots highlight those substances that were significantly down-regulated. The  
239 substances without significant difference between the two groups are represented by  
240 gray dots. To evaluate the variation between the two honey samples and simplify the  
241 data management, PCA was used. The raw data of 3,060 MFs were subjected to PCA  
242 algorithm in the MPP software (Figure. 1. C). The 2D PCA shown represents 67.99%  
243 of the total variation. The first principle component (PC1) accounted for 60.29% of the  
244 total data variability, while the second one accounted for 7.7%. The distribution areas  
245 of the two samples are clearly differentiated. IMH is mainly distributed in the positive  
246 axis of PC1, while MH is mainly in the negative axis of PC1.

#### 247 Targeted study

248 Twenty types of phenolic compounds were qualitatively analyzed by HPLC-Q-  
249 TOF/MS (Figure. 2 & Table 2). Further quantitative analysis showed that except for  
250 vanillic acid and syringic acid, the concentrations of 3, 4-dihydroxybenzoic acid,  
251 chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, 3-O-acetylpinobanksin,  
252 quercetin, hesperitin, pinobanksin, naringenin, galangin, luteolin, kaempferol, apigenin,  
253 pinocembrin, 3-(3, 4-Dimethoxyphenyl)-2-propenoic acid, chrysin, caffeic acid, and  
254 phenethyl ester in MH was significantly higher than that of IMH. Among them,  
255 kaempferol, apigenin, pinocembrin, 3-(3, 4-dimethoxyphenyl)-2-propenoic acid,  
256 chrysin and caffeic acid phenethyl ester were only detected in MH.

#### 257 *Comparison on the anti-bacterial activity*

258 We measured the anti-bacterial activity of honey solution against *Escherichia coli*,  
259 *Staphylococcus aureus* and *Bacillus subtilis* (Table 3). The result revealed that the zones

260 of inhibition of MH and IMH on *Escherichia coli* and *Staphylococcus aureus* were  
261 19.47±0.31 mm, 14.13±0.68 mm and 17.29±0.78 mm, 12.80±0.98 mm, respectively.  
262 However, neither of them showed any obvious inhibitory effect on *Bacillus subtilis* as  
263 no zones were observed. The zones of inhibition were significantly higher for the MH  
264 than for the IMH (both P<0.05), indicating that MH has a stronger bacteriostatic effect  
265 than IMH.

#### 266 *Comparisons of the anti-oxidant activities*

267 The experimental results (Table 4) of oxidation resistance showed the content of  
268 total phenolics and total flavonoids in the extracts of honey. The MH and IMH  
269 samples contained total phenolics of 12.99 ± 0.19 mg gallic acid equivalent (GAE) per  
270 gram and 12.20 ± 0.16 mg gallic acid equivalent (GAE) per gram, respectively, and  
271 total flavonoids as 3.53± 0.07 mg quercetin equivalent (QE) per gram and 3.41 ± 0.01  
272 mg quercetin equivalent (QE) per gram, respectively. The reduction capacity was 36.97  
273 ± 0.53 mg Trolox equivalent per gram and 28.41 ± 0.76 mg Trolox equivalent per gram  
274 in MH and IMH, respectively. DPPH and ABTS<sup>+</sup> Free radical scavenging power were  
275 21.89 ± 0.08 mg Trolox equivalent per gram and 19.60 ± 0.36 mg Trolox equivalent per  
276 gram, 37.82 ± 0.90 mg Trolox equivalent per gram and 32.30 ± 0.81 mg Trolox  
277 equivalent per gram.

278 Subsequently, cell experiments were conducted to further study the anti-oxidant  
279 effect of honey. The results showed that when the concentration of H<sub>2</sub>O<sub>2</sub> was 500 µM,  
280 the anti-oxidative activity of L929 cells was significantly reduced. However, the honey  
281 extract had no toxic effect in the range of test concentrations and honey extract

282 treatment significantly improved the proliferation activity of cells stimulated by H<sub>2</sub>O<sub>2</sub>  
283 (Figure. 3). MHE concentration of 400 µg/mL significantly increased the expression of  
284 anti-oxidant genes HO-1, TXNRD, GCLM and NQO1 (Figure. 4). IMHE was only  
285 effective when the concentration of IMHE was 600 µg/mL, and the expression of anti-  
286 oxidation gene NQO1 did not significantly promote the effect.

287

## 288 **Discussion**

289 To determine whether immaturity of honey might adversely affect honey quality,  
290 we performed a comparative study of the differences between the capped and uncapped  
291 honeys from the same botanic source (*Brassia campestris L*). We found notable  
292 physicochemical and bioactive differences between immature and mature honeys.

293 The physicochemical indices, including water, sugar, acidity, and HMF, have been  
294 previously investigated.<sup>16</sup> These are the basic indicators to characterize the quality of  
295 honey. Studies have shown that the physicochemical parameters of honey can be  
296 utilized to distinguish between mature and immature acacia honey.<sup>17</sup>

297 The water content of honey represents a highly important quality parameter for  
298 the its shelf life during storage. The origin of honey, nectars normally, contain more  
299 than 50% water but bees will further dehydrate the honey in the comb environment.<sup>18</sup>  
300 Therefore, early harvest of the immature honey leads to high moisture content.<sup>19</sup> High  
301 water content increases the possibility of honey being fermented during long storage  
302 periods.<sup>20</sup> The average water content of our MH samples (18.31 ± 1.52 g per 100 g)  
303 were below the required threshold standard of the European Regulations of Quality



304 (no more than 20 g per 100 g). The average water content of IMH samples was well  
305 above this standard ( $31.20 \pm 1.81$  g per 100 g) suggesting reduced quality and  
306 increased possibility of fermentation.

307 Increased free acidity is an important indicator of microbial spoilage and  
308 freshness of honey. When acidity values are above the standard limits, it indicates  
309 sugar fermentation due to the formation of acetic acid by alcohol hydrolysis.<sup>21</sup>  
310 Depending on the flower source or geographic area, the free acidity of honey varies.  
311 As reported in a previous study, honey's acidity ranges from 9.7 to 29.5 meq/kg.<sup>22</sup> In  
312 our study, the free acidity of capped rapeseed honey was  $13.67 \pm 1.88$  meq/kg, whereas  
313 for uncapped it one is  $19.9 \pm 0.42$  meq/kg. Both results were below the required  
314 standard (less than 50 meq/kg).<sup>23</sup> This shows that, the acidity of honey not only  
315 depends on nectar source species but is also affected by maturity.

316 HMF represents an indicator of honey freshness and authenticity whereas high  
317 concentrations of HMF in honey indicates overheating and poor storage conditions or  
318 adulteration of the honey with inverted syrup.<sup>24</sup> Honey storage at 35°C causes an  
319 increase of HMF that exceeds the allowed limit (40 mg/Kg).<sup>25</sup> However, HMF was  
320 not detected in our study samples, which means that the honey samples were fresh,  
321 but the immature uncapped honey would need to be further dehydrated including a  
322 heating process which may increase HMF levels.

323 Sugar is the main ingredient in all honeys, with concentrations of up to 80%, and  
324 explains why honey is the oldest natural sweetener.<sup>26</sup> The sugar content of honey  
325 varies from harvest time, due to the flutter of the wings of the bees or the variance

326 among the nectars.<sup>27</sup> The most abundant sugars in honey are fructose and glucose,  
327 with higher quantities of fructose in the majority of honeys.<sup>28</sup> The percentage of  
328 fructose and glucose in our samples ranged from 15.5-49.3% and 18.2–48.0%,  
329 respectively. Sucrose was not detected or detected in very low amounts in the honey  
330 samples, this is not surprising since sucrose is broken down into glucose, fructose and  
331 other monosaccharide by enzymes secreted by bees during honey maturation process.  
332 Rapeseed honeys normally contain more glucose than fructose, but in this study,  
333 glucose content was found to be at lower concentrations than fructose. This may be  
334 due to the different geographical origin and the local climate. The results also suggest  
335 that a higher percentage of fructose may be produced as honey is matured in the hive  
336 for a long time. Mature honey has been shown to have lower water content, higher  
337 fructose content and lower acidity than immature honey, and therefore MH appears to  
338 be of better quality.

339 Recently, metabolic profiling methods have been robustly applied to detect the  
340 intrinsic similarities and differences in metabolites within biological samples.<sup>29</sup> In the  
341 present study, the Mass Profiler Professional (MPP) software was applied in the  
342 analysis of the chromatographic data, which enabled us to compare accurately,  
343 comprehensively and quickly the major constituents between MH and IMH samples.<sup>30</sup>  
344 This MPP analysis has already been used for screening and development of drugs and  
345 food inspection, the results of which have been well-recognized in related fields. The  
346 method has been confirmed to be precise, accurate and sensitive enough for untargeted  
347 analysis.<sup>30,31</sup> The present study is the first application of MPP technology in determining

348 honey maturity. We analyzed 18 batches of honey samples collected from three different  
349 hives. To ensure comparability each comb analysed contained both immature  
350 (uncapped) and mature (capped) honey, during a single rapeseed blossom season. We  
351 also performed multivariate statistical analysis for classification, prediction, and  
352 characterization of marker compounds. Among them, some metabolites, including  
353 organic acids, flavonoids, polyphenols, terpenes and others have been reported from  
354 honey.<sup>32,33</sup> We investigated the differences in the metabolite composition in honeys  
355 under different maturation conditions. As seen in PCA-score plots (Figure. 1. C), IMH  
356 samples separate from MH samples, indicating a large difference between the two  
357 groups of samples. A volcano plot representing the filtered data is shown in Figure. 1.  
358 B The compounds found at significantly ( $P < 0.05$ ) higher levels in IMH than MH  
359 samples were organic acids (benzoic acid, linalool, sinapic acid and ganoderic acid *etc.*),  
360 alcohols, some derivatives of acids (ethyl gallate, levistilide), some glycosides, plant  
361 alkaloids, and very small amounts of phenolic compounds such as vanilic acid and  
362 eugenic acid. The compounds found at significantly ( $P < 0.05$ ) lower levels were caffeic  
363 acid, 3, 4-dihydroxybenzoic acid, chlorogenic acid and common phenolic substances.

364 To further understand the material differences between the two kinds of honey, we  
365 chose to analyze the polyphenols that are major active ingredients in honey. A total of  
366 29 types of flavonoids and phenolic acids were studied of which 20 were detected.  
367 These compounds were selected as they were predominately the active constituents in  
368 honey as well as propolis. We have previously established accurate quantification  
369 methods for these chemicals.<sup>14,34</sup> Average concentrations of these polyphenolic

370 compounds varied greatly among samples (between  $0.38 \pm 0.01$  to  $158.09 \pm 2.89$   
371  $\mu\text{g}/100$  g honey). Six of these compounds were only detected in MH, including  
372 kaempferol, apigenin, pinocembrin, 3-(3, 4-dimethoxyphenyl)-2-propenoic acid,  
373 chrysin and caffeic acid phenethyl ester. Interestingly, with the exceptions of vanillic  
374 acid and syringic acid, the content of the remaining 12 substances in MH were  
375 significantly higher than those in IMH. These polyphenols are derived from plants, and  
376 are known as the key contributors to the honey's color and taste, as well as its biological  
377 activities.<sup>35</sup> Of course, phenols can vary depending on nectar plant, bee species and  
378 geographic source.<sup>36,37</sup> It is well documented that plant phenolic metabolites change by  
379 the action of bee enzymes in honey. The results from the present study show significant  
380 changes, which might be due to interaction with some substances in the beehive, like  
381 hydrolysis from glycosides to give rise to aglycones. Nevertheless, we only analyzed  
382 honey of unifloral origin and it would be interesting to further compare differences  
383 between mature and immature honeys from other nectar sources and over different  
384 seasons.

385       The anti-microbial activity of honey is clearly established and honey could provide  
386 a potential alternative to antibiotics.<sup>39</sup> The possible underlying mechanism of action  
387 relies on the ability of honey to generate hydrogen peroxide by the bee-derived enzyme  
388 glucose oxidase.<sup>40,41</sup> However, other factors may also contribute to its antimicrobial  
389 activity such as high osmotic pressure, acidic environment, low protein content, high  
390 carbon to nitrogen ratio, low redox potential (due to the high level of reducing sugars),  
391 and a level of viscosity that limits dissolved oxygen and other chemical

392 agents/phytochemicals. Another potential contributor is the complex composition of  
393 honey, which has more than 181 constituents.<sup>42</sup> These include terpenes, pinocembrin,  
394 benzyl alcohol, 3, 5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl-3, 5-  
395 dimethoxy-4-hydroxybenzoate (methyl syringate), 2-hydroxy-3-phenylpropionic acid,  
396 2-hydroxybenzoic acid, 3, 4, 5-trimethoxybenzoic acid, and 1, 4-dihydroxybenzene.  
397 Consistent with previous studies, we found that honey exhibits a bacteriostatic against  
398 several pathogens, such as *Staphylococcus aureus* and *Escherichia coli*. *E. coli* is a  
399 Gram-negative bacterium that is pathogenic to human and animals and can cause  
400 diarrhea and sepsis in children, travelers, piglets and chickens.<sup>43</sup> *S. aureus* is a  
401 representative of gram-positive bacteria and infection can cause serious illness in  
402 humans.<sup>44</sup> Our results showed that the zones of inhibition of *E.coli* and *S. aureus* by  
403 mature rapeseed honey were bigger than the immature rapeseed honeys, thus  
404 demonstrating that MH has a stronger bacteriostatic effect than IMH. Nevertheless,  
405 both samples had no obvious inhibitory effect on *Bacillus subtilis*. *B. subtilis* is a  
406 multifunctional probiotic and is beneficial for human digestion and absorption. It  
407 produces subtilisin, polymyxin and other active substances to inhibit intestinal pathogenic  
408 bacteria.<sup>45</sup>

409 Honey works as an abundant source of natural anti-oxidants which play an  
410 important role in food preservation and human health.<sup>12</sup> Anti-oxidant substances have  
411 different mechanisms, such as reducing the damaging effects of reactive oxygen and  
412 reactive nitrogen species, inhibiting the effects of enzymes that produce superoxide  
413 anions, promoting metal chelation and free radical chain reaction, and inhibiting the

414 formation of active oxidants.<sup>37</sup> In the present study, three standard spectrophotometric  
415 methods are used for comparing the *in vitro* anti-oxidant effects of MH and IMH  
416 samples: The DPPH test and ABTS<sup>+</sup> test for radical scavenging activity and the Ferric  
417 reducing ability of plasma (FRAP) method for their reducing power.<sup>15</sup> The main anti-  
418 oxidants in honeys are polyphenols, including phenolic acids and flavonoids. According  
419 to previous studies, the total phenolic content of honey is uncertain, ranging from 0.205  
420 mg GAE/g to 1.877 mg GAE/g honey, while among rapeseed honey, it ranges from  
421 0.205 mg GAE/g to 0.311 mg GAE/g honey.<sup>15,40</sup> In this study, we studied the total  
422 phenolic content of honey extracts, producing results of  $12.99 \pm 0.19$  and  $12.20 \pm 0.16$   
423 mg GAE/g honey extracts for MH and IMH, respectively. Although these values are  
424 fall within a certain range with previous literature, our results are significantly higher  
425 than previously published data.<sup>46</sup> An explanation for this may be that we extracted the  
426 honey before testing it. The content of total phenols in the honey polyphenol extract of  
427 mature rapeseed honey ( $12.99 \pm 0.19$  mg GAE/g extract) was significantly higher than  
428 that of immature rapeseed honey ( $12.20 \pm 0.16$  mg GAE/g extract). However, there was  
429 no significant difference in the content of total flavonoids. Rapeseed honey from  
430 different geographical sources has been shown to possess different anti-oxidant  
431 capacities. Piotr Marek Kuś et al.<sup>47</sup> studied the anti-oxidant capacity of 10 kinds of  
432 rapeseed honey from 8 regions in Poland, finding that the FRAP level was 1.0-1.8  
433 (mmol Fe<sup>2+</sup>/kg), and the average level was  $1.3 \pm 0.3$ . DPPH level was 0.3-0.5 (mmol  
434 TEAC/kg), average level was  $0.4 \pm 0.1$  (mmol TEAC/kg). The FRAP and DPPH values  
435 of MH and IMH samples in our study were smaller than Piotr Marek Kuś et al reported,

436 but the MH has a stronger anti-oxidant activity than the IMH.

437 Honey has a regulatory effect on cell growth and proliferation, metabolism and  
438 anti-oxidant enzymes, and has a protective effect on cell damage caused by adverse  
439 stimulation.<sup>11</sup> The mechanisms by which honey influences the biological activity of  
440 cells is complex .<sup>49,50</sup> In this study, a cell oxidative stress model was applied in mouse  
441 fibroblasts (L929) stimulated by hydrogen peroxide as previously established.<sup>51</sup> Firstly,  
442 the concentration of H<sub>2</sub>O<sub>2</sub> was determined by toxicity testing, as shown in the figure 3.  
443 The reproductivity of the cultured cells can be significantly reduced when treated with  
444 500 μM H<sub>2</sub>O<sub>2</sub>, but the honey extract had no toxic effect on cells in the range of tested  
445 concentrations. Then cells were pretreated with honey extract prior to 500 μM H<sub>2</sub>O<sub>2</sub>  
446 treatment. Our results demonstrated that the honey extract could significantly improve  
447 the cell growth activity. MH showed a positive effect at the concentration of 400 μg/mL,  
448 while for IMH is the required concentration was 600 μg/mL. This suggests that honey  
449 can counteract the cell damage caused by oxidative stress, with the effect of mature  
450 honey more potent.

451 In the meantime, we examined the expression of antioxidant genes (*HO-1*, *TXNRD*,  
452 *GCLM*, and *NQO1*) in cells. The results showed significantly increased expression of  
453 anti-oxidant genes in the MH-pretreated cells. However, the effect of IMH was weak  
454 and had no significant effect on *NQO1* gene expression. Heme oxygenase 1 (HO-1),  
455 catalyzes the decomposition of heme into a series of anti-oxidant and anti-inflammatory  
456 molecules that prevent oxidation;<sup>52</sup> NQO1 catalyzes double electron reduction to  
457 reduce oxidative damage;<sup>53</sup> GCLM is a subunit of glutamic acid and cysteine synthase,

458 the most important genes in the cellular anti-oxidant defense mechanism;<sup>54</sup> TXNRD  
459 (thioredoxin reductase) is involved in many redox reactions in vivo.<sup>55</sup> These anti-  
460 oxidant genes are important regulators of NRF-2 signaling pathway.<sup>56</sup> The NRF-2  
461 signaling pathway regulates the transcriptional expression of many proteins with  
462 detoxification and anti-oxidant defense functions. Our results suggest that honey may  
463 affect the cellular oxidative stress response by affecting the NRF-2 signaling pathway.

464 This study performed analysis of mature and immature honey using untargeted and  
465 targeted methods, and determined their anti-bacterial and anti-oxidant activity *in vitro*.  
466 The results demonstrated that the harvest of honey before the maturity stage can have  
467 profound impacts upon its quality. Our study demonstrated using metabolomics data  
468 analysis the possibility to that mature honey and immature honey could be distinguished  
469 by the metabolite differences between them by means of metabolomics data analysis.  
470 Untargeted substance analysis based on Mass Profiler Professional software explains  
471 the difference between the two from a macro perspective. Further in-depth analysis of  
472 target substance research indicates that effective and beneficial substances are more  
473 abundant in mature honey than in immature honey. This is the first time that  
474 metabolomics analysis technology was applied to the study of honey quality. Results  
475 from in vitro anti-bacterial and anti-oxidant experiments showed that mature capped  
476 honey is more effective in inhibiting proliferation of *E. coli* and *S. aureus*, and may  
477 protect mice skin fibroblast L929 cells from the damage of free radicals by enhancing  
478 the expression of anti-oxidant related genes after H<sub>2</sub>O<sub>2</sub> stimulation. In conclusion,  
479 mature honey has a greater value.



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489 **Conflict of interest**

490 The authors declare that they have no conflict of interest.

491

492 **Supporting Information.**

493 Supplemental Table 1: Sequences of the primers used for qRT-PCR

494

495

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673

674 **Figure Captions**

675 **Figure 1** Discrimination of mature capped honey and immature uncapped honey (MH  
676 represents mature honey; IMH represents immature honey). A: Venn diagram of  
677 untargeted analysis of MH and IMH with a filtration of samples frequency (70%). The  
678 number in the picture represents the number of species of matter. B: Volcano plot of the  
679 honey different metabolites for group MH vs IMH ( $P < 0.05$ ). C: PCA scores plot of MH  
680 and IMH.

681 **Figure 2** Total ion chromatography of honey extracts with negative scanning mode in  
682 HPLC-Q-TOF-MS. Red line represents mature honey (MH); Green line represents  
683 immature honey (IMH). The samples for 0-1 min are discarded without mass  
684 spectrometry.

685 **Figure 3** Effect of  $H_2O_2$  and honey extracts on L929 cells viability. (A). Cells were  
686 pretreated with/without the indicated concentrations of  $H_2O_2$  (300  $\mu M$ -600  $\mu M$ ) and  
687 honey extracts (0  $\mu g/mL$ -600  $\mu g/mL$ ) for 24 h. (B)(C). Cells were pretreated  
688 with/without the different concentrations of MHE/IMHE for 2 h and then stimulated  
689 with 500  $\mu M H_2O_2$  for 24 h. ★ indicates the control group for significance analysis. Each  
690 result was expressed as the mean  $\pm$  SD ( $n = 3$ ); \* $P < 0.05$  versus the control group (★);  
691 \*\* $P < 0.01$  versus the control group (★); \*\*\* $P < 0.001$  versus the control group (★).

692 **Figure 4** Effect of honey extracts on the expression of antioxidant related genes in  $H_2O_2$   
693 stimulated cells. L929 cells were pretreated with or without the indicated concentrations  
694 of MHE/IMHE for 2 h and were then stimulated with 500  $\mu M H_2O_2$  for 6 h. The relative  
695 mRNA expression of *HO-1*(A), *TXNRD* (B), *GCLM* (C) and *NQO1* (D) were

696 determined using qRT-PCR. Each result was shown as the mean  $\pm$  SD (n = 3). \*\*P <  
697 0.01 versus the untreated group (★), \*\*\*P < 0.001 versus the untreated group (★).  
698

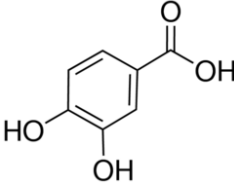
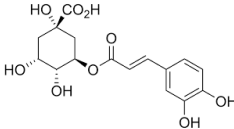
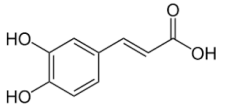
## Tables

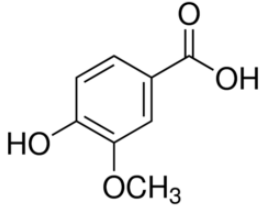
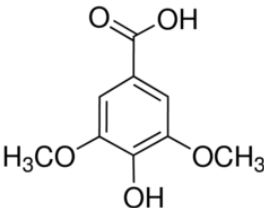
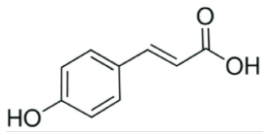
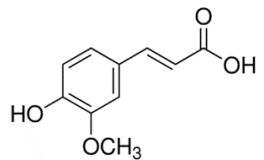
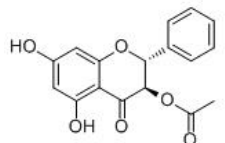
**Table 1 Parameters of Mature and Immature Honey\*.**

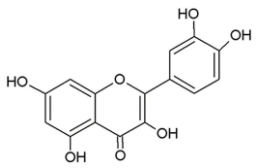
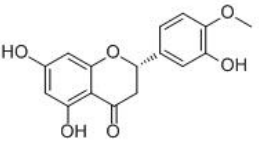
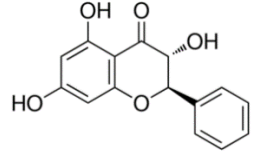
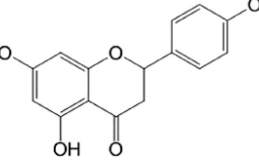
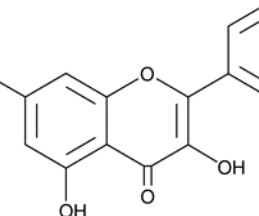
Parameter	MH	IMH
Fruuctose%	36.40± 0.37 <sup>a</sup>	30.86 ± 0.64 <sup>b</sup>
Glucose%	34.49± 2.17 <sup>a</sup>	30.14 ± 0.72 <sup>a</sup>
Sucrose%	2.33 ± 0.17 <sup>a</sup>	1.11 ± 0.73 <sup>a</sup>
Water%	18.31 ± 1.52 <sup>a</sup>	31.20 ± 1.81 <sup>b</sup>
Acidity		
meq/kg	13.67 ± 1.88 <sup>a</sup>	19.9 ± 0.42 <sup>b</sup>
HMF	ND	

\* In each column, different letters (a, b) mean significant differences ( $p < 0.05$ ). ND means not detected.

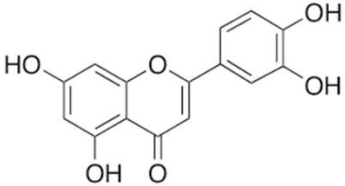
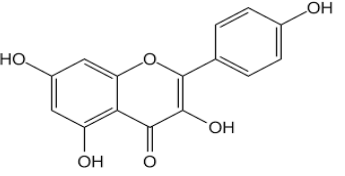
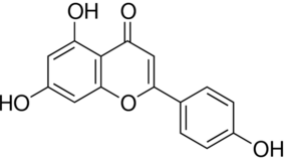
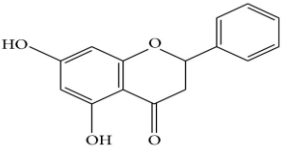
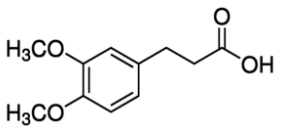
**Table 2 HPLC-Q-TOF MS Analysis of Major Phenolic Compounds and Relative Occurrence in MH and IMH\*.**

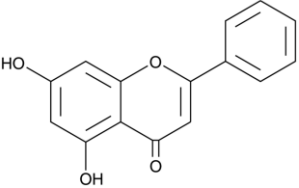
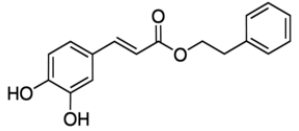
Compounds	Molecular formula	Molecular weight	Molecular formulae	[M-H] <sup>-</sup> , <i>m/z</i>	RT/mi n	μg/100gMH	μg/100gIMH	LOD μg/100g	R <sup>2</sup>
3,4-Dihydroxybenzoic acid	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>	154.12		153.0193	4.596	7.20±0.05 <sup>a</sup>	3.05±0.05 <sup>b</sup>	0.025	0.999
Chlorogenic acid	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	354.31		353.0878	5.510	11.70±0.20 <sup>a</sup>	4.80±0.09 <sup>b</sup>	0.006	0.990
Caffeic acid	C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>	180.16		179.0350	6.035	22.86±0.88 <sup>a</sup>	5.37±0.05 <sup>b</sup>	0.023	0.992

Vanillic acid	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	168.15		167.0350	5.950	51.17±0.58 <sup>b</sup>	60.10±0.25 <sup>a</sup>	0.250	0.99	5
Syringic acid	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>	198.17		197.0455	6.169	6.81±0.04 <sup>b</sup>	14.68±0.12 <sup>a</sup>	0.147	0.99	0
p-Coumaric acid	C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>	164.16		163.0401	6.804	9.51±0.52 <sup>a</sup>	1.38±0.04 <sup>b</sup>	0.013	0.99	0
Ferulic Acid	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	194.18		193.0506	6.950	19.84±1.10 <sup>a</sup>	4.36±0.13 <sup>b</sup>	0.043	0.99	8
3-O-Acetylpinobanksin	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	314.00		313.0718	11.561	13.53±0.40 <sup>a</sup>	0.38±0.01 <sup>b</sup>	0.008	0.99	4

Quercetin	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	302.24		301.0354	8.743	134.16±2.41	58.94±0.63 <sup>b</sup>	0.057	0.99
						a			1
Hesperitin	C <sub>16</sub> H <sub>14</sub> O <sub>6</sub>	302.28		301.0718	8.939	158.09±2.89	76.32±0.83 <sup>b</sup>	0.012	0.99
						a			1
Pinobanksin	C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>	272.25		271.0612	8.928	25.74±0.20 <sup>a</sup>	0.84±0.01 <sup>b</sup>	0.027	0.99
									3
Naringenin	C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>	272.25		271.0612	9.098	23.39±0.33 <sup>a</sup>	0.88±0.01 <sup>b</sup>	0.027	0.99
									3
Galangin	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	270.24		269.0455	9.122	8.42±0.24 <sup>a</sup>	3.63±0.01 <sup>b</sup>	0.018	0.99
									0



Luteolin	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	286.24		285.0405	9.952	67.32±1.09 <sup>a</sup>	34.82±0.57 <sup>b</sup>	0.062	0.99
									0
Kaempferol	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	286.24		285.0405	11.391	25.44±0.46	ND	0.078	0.99
									8
Apigenin	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	270.24		269.0455	7.792	15.24±0.19	ND	0.039	0.99
									3
Pinocembrin	C <sub>15</sub> H <sub>12</sub> O <sub>4</sub>	256.25		255.0663	12.037	10.95±0.31	ND	0.036	0.99
									2
3-(3,4-Dimethoxyphenyl)-2-propenoic Acid	C <sub>11</sub> H <sub>12</sub> O <sub>4</sub>	208.21		207.0663	12.184	19.60±0.23	ND	0.166	0.99
									1

Chrysin	C <sub>15</sub> H <sub>10</sub> O <sub>4</sub>	254.24		253.0506	12.612	18.83±0.97	ND	0.011	0.99
Caffeic acid phenethyl ester	C <sub>17</sub> H <sub>16</sub> O <sub>4</sub>	284.31		283.0976	9.793	12.89±0.26	ND	0.025	0.99

\* Detected in negative ionization mode. In each column different letters (a, b) mean significant differences ( $p < 0.05$ ). ND means not detected.

**Table 3 Zones of inhibition of MH and IMH\*.**

	Zones of inhibition (mm)				
	Ampicillin(5µg/mL)	Phenol(10%)	50%MH	50%IMH	Water
<i>E.coli</i>	17.69±0.43	15.40±0.57	19.47 <sup>a</sup> ±0.31	17.29 <sup>b</sup> ±0.78	--
<i>S.aureus</i>	29.23±0.62	14.40±0.69	14.13 <sup>a</sup> ±0.68	12.8 <sup>b</sup> ±0.98	--
<i>B.subtilis</i>	0.50±0.08	20.90±0.29	--	--	--

\* In each column different letters (a, b) mean significant differences ( $p < 0.05$ ). -- means that there is no observed bacteriostatic zone.

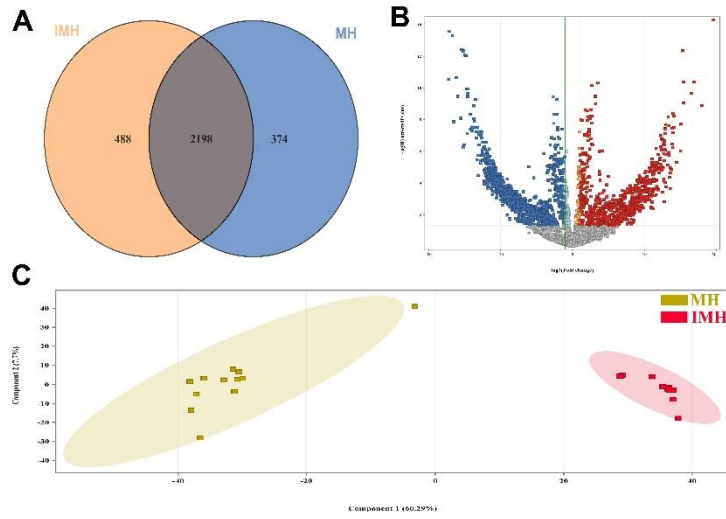
**Table 4 Antioxidant Activity of Mature Honey and Immature Honey. Including Radical Scavenging Capacity, Reducing Power, and Total Phenols and Flavone of MH and IMH\*.**

	FRAP		ABTS		DPPH		Total	Total
	IC50	mg Trolox	IC50		IC50		phenols	flavonoids
	mg/mL	/g	mg/mL	mgTrolox/g	mg/mL	mgTrolox/g	mgGAE/g	mgQE/g
MH	1.69±0.02	36.97±0.53 <sup>a</sup>	0.86±0.02	37.82±0.90 <sup>a</sup>	2.26±0.01	21.89±0.08 <sup>a</sup>	12.99±0.19 <sup>a</sup>	3.53±0.07 <sup>a</sup>
IMH	2.21±0.06	28.41±0.76 <sup>b</sup>	1.01±0.06	32.30±0.81 <sup>b</sup>	2.53±0.05	19.60±0.36 <sup>b</sup>	12.20±0.16 <sup>b</sup>	3.41±0.01 <sup>a</sup>
Vc (µg/mL)	35.07±0.02		16.76±0.06		28.01±0.02			

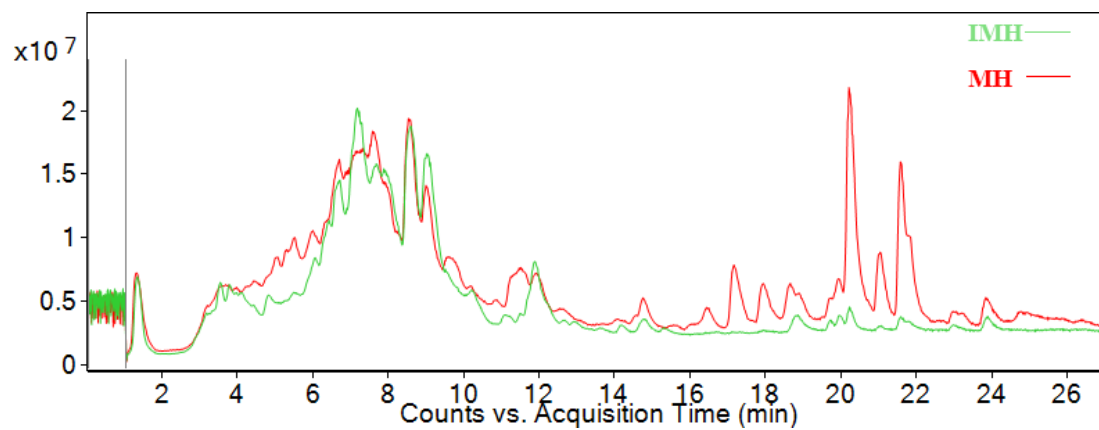
\* In each column different letters (a, b) mean significant differences ( $p < 0.05$ ). IC50 means the sample concentration providing 0.5 of absorbance was determined by a linear curve established by mass concentration and absorbance. Meanwhile all the results were expressed as equivalent of the corresponding standard reference (mg Trolox equivalent per gram (mg Trolox/g); mg gallic acid equivalent per gram (mg GAE/g); mg quercetin equivalent per gram (mg QE/g)).

# Figure graphics

## Figure 1



**Figure 2**



**Figure 3**

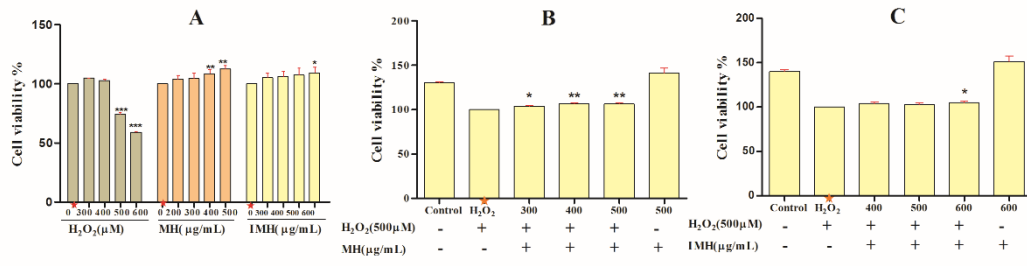
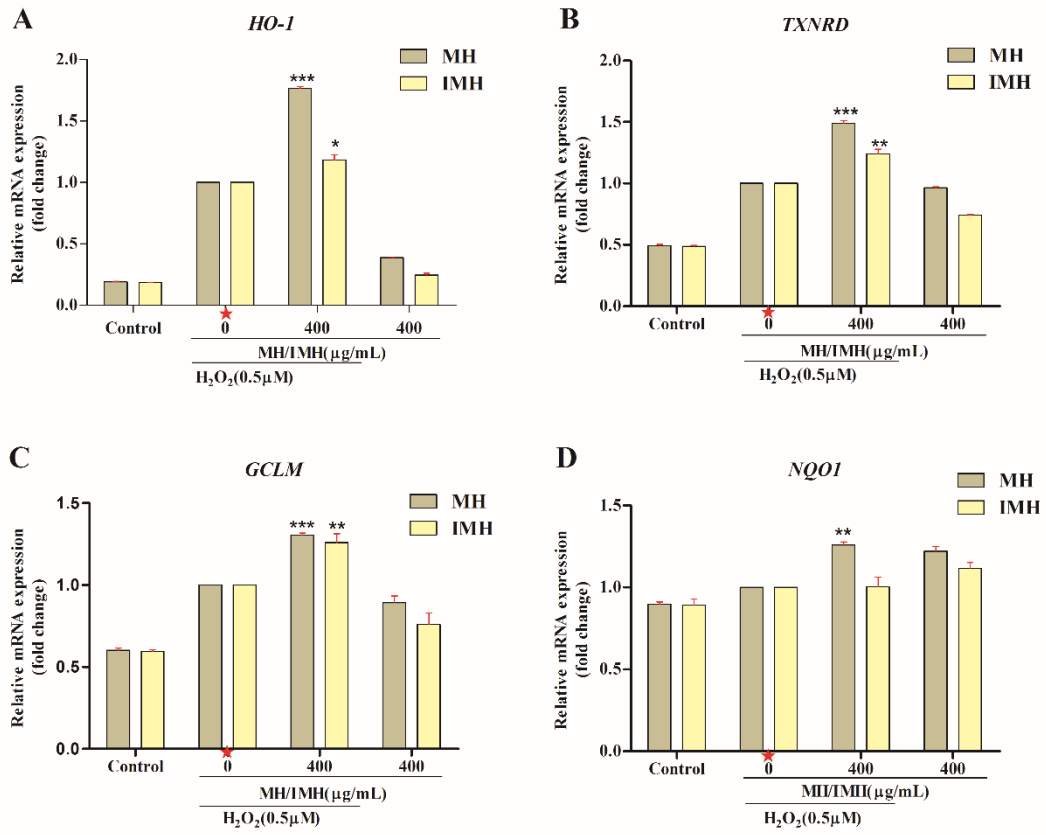
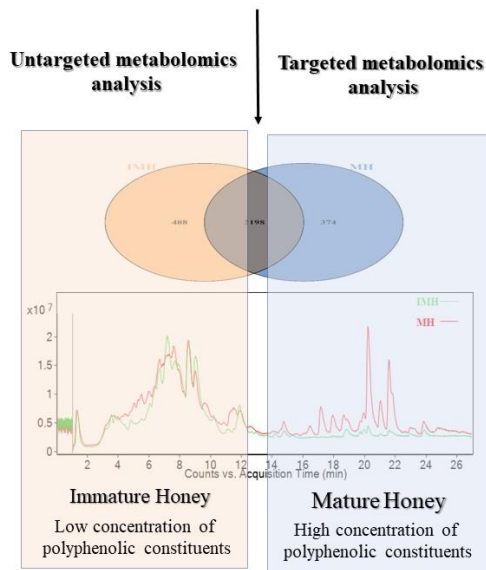


Figure 4





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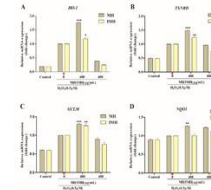


**MH had stronger anti-bacterial effects**

	Inhibition zone(mm)				
	Ampicillin(5µg/mL)	Phenol(10%)	50%MH	50%IMH	Water
E.coli	17.69±0.020	15.4±0.01	19.47±0.002	17.29±0.003	0
S.aureus	29.23±0.010	14.4±0.009	14.13±0.004	12.8±0.006	0
B.subtilis	0.5±0.001	20.9±0.007		0	0

**MH had stronger free radical scavenging effects**

FRAP,  
ABTS,  
DPPH,  
Total phenols,  
Total flavonoids  
MH > IMH



**MH had better anti-oxidative effects in H<sub>2</sub>O<sub>2</sub> induced L929 cellular oxidative damages**

