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Pollen diversity and secondary metabolites in honey produced by *Apis dorsata binghami* from Central Sulawesi, Indonesia

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Abstract
Honey produced by the giant honeybee (*Apis dorsata binghami*) is considered as highly nutritious natural product and has long been used as health supplement. This type of honey is collected traditionally by honey hunters using crude method from forest. This study aimed at determining the diversity of pollen and secondary metabolite compounds in honey samples produced by giant honeybee collected from four regencies in Central Sulawesi. Honey samples were collected from Donggala, Banggai, Morowali, and Parigi Moutong. Modified acetolysis method was used for preparing pollen microscopic slides, while TLC method was performed for the detection of secondary metabolite compounds. The results showed that honey samples varied in their pollen diversity, with the number of pollen types found in honey sample from Donggala was 33, Banggai was 36, Morowali was 30, and Parigi Moutong was 57. All honey samples contained five classes of secondary metabolite compounds namely alkaloid, phenolic, flavonoid, tannin, and terpenoid. From this melissopalynological research, it could be concluded that honey samples produced by giant honeybee from Central Sulawesi are of good quality based on their pollen diversity and the content of secondary metabolite compounds.

Keywords: Melissopalynology, honey quality, *Apis dorsata*

Introduction
Honey is a liquid natural product mainly containing sugar which has been used for long time as food supplement for improving health. Honey is produced by honeybees from flower nectar or other parts of plants through series of processes by bee colony. The chemical composition of nectar is generally consisted of water, carbohydrates, lipids, amino acids, and secondary metabolites [1]. Accordingly, honey contains various chemicals of natural origin, including carbohydrates, lipids, proteins, amino acids, minerals, vitamins, enzymes, organic acids, and plant secondary metabolite compounds [2].

There are many varieties of honey that vary on their physicochemical properties depending on the honeybee species producing them [3], and their geographical origin [4]. Each type of honey varies in secondary metabolites content depending on the type of plants visited by honeybees [5, 6], since secondary metabolites found in honey come from flower nectars collected by honeybees [7]. The honeybees collect nectar as well as pollen during their visit to flowers, and bring the pollen to their hive as food [5, 8]. Since honeybees visit various plants, the diversity of nectar and pollen collected by the bee causes variations in secondary metabolites found in honey, and these variations greatly affect the physicochemical properties of honey [9]. Melissopalynological study, that is the analysis of pollen content in honey, become a reliable method in determining plants visited by honeybees, and thus provide evidence for ensuring botanical and geographical origin of honey [10, 11, 12].

One of popular honey variants is those produced by a colony of giant honeybee (*Apis dorsata*). This species has unique behavior in building nest, that is build the nest in open areas on sturdy branches of high trees, mostly more than 10 m high in the forests with dense vegetation [13]. Melissopalynological studies on honey produced by *A. dorsata* are still limited compared to those produced by *A. mellifera* and *A. cerana* because the aggressive nature of *A. dorsata* which makes it difficult to collect their honey. Melissopalynological studies on *A. dorsata* in Indonesia are still very limited, and previous studies on *A. dorsata* honey were commonly carried out in other countries, such as in India [14, 15, 16] and Malaysia [17].

The objective of this study was to reveal the diversity of pollen and secondary metabolites in honey produced by A. *dorsata binghami*, a subspecies of *A. dorsata* naturally occur in Sulawesi and its surrounding islands. The results of this study are expected to provide scientific evidence to support the use of honey as health supplement based on pollen and secondary metabolites content.

The availability of scientific evidence form this study will be beneficial for honey industry in terms of promoting *A. dorsata* honey as one of the forest products from South Sulawesi.

Materials and Methods

Honey samples were collected from four regencies in Central Sulawesi Province, namely Donggala, Banggai, Morowali, and Parigi Moutong (Figure 1). Fresh honey samples were obtained by directly squeezing the honeycomb manually, collected in glass bottles, and stored in refrigerator before the laboratory analysis processes.

The procedure for preparing microscopic slides of pollen was done using acetolysis method with some modifications [18]. The first step was dilution of 5 ml of honey sample in 7 ml of distilled water, and the mixture was centrifuged at 1700 rpm for 5 minutes. After the supernatant was removed, the remaining pellets consisted of pollen grains and some organic materials was treated with 7 ml of 38% hydrochloric acid solution, mixed well, and incubated in a water-bath chamber at a temperature of 100°C for 30 minutes. The solution was then centrifuged at 1700 rpm for 5 minutes, and the supernatant was removed. The pellet was then neutralized by adding 7 ml of 10% potassium hydroxide solution. After being centrifuged at 1700 rpm for 5 minutes, the supernatant was removed and the pellets was washed with distilled water several times until the solution became clear, indicated that all the potassium hydroxide and organic materials were washed away. Staining process was done by adding 1 ml of 1% safranin into the sample, and kept at room temperature for 24 hours. The stained pellet was washed using distilled water and then centrifuged at 1700 rpm for 5 minutes to eliminate the remaining safranin stain. A drop of pellet containing pollen grains was placed on microscopic slides, left at room temperature for 5-10 minutes, and then a drop of absolute ethanol and a drop of glycerin were added, before a glass cover was put firmly over the sample.

(Google map; https://www.google.co.id/maps/place/Central+Sulawesi/ accessed on 22 October 2019)

Fig 1: Location of four regencies in Central Sulawesi where honey samples were collected

Examination on morphological characters of pollen grains was done under a Boeco BM-180 light microscope equipped with Miconos OptiLab digital camera for microscopy on a magnification of 10x40. Pollen identification was carried out using determination keys and photographs on Pollen Flora of Taiwan [19] and the Australian Pollen and Spore Atlas website (http://apsa.anu.edu.au).

The analysis of secondary metabolite compounds was performed using thin layer chromatography (TLC) method. Each honey sample was extracted using a liquid-liquid partition method [20]. The procedure was started by placing 100 ml of honey into a separating funnel and then 100 ml of ethyl acetate was added into it. The mixture was shaken and the resulted solution was allowed to settle down until two layers of liquid were formed. The upper layer was taken and put into the evaporator. The resulted product of evaporation process was then spotted onto the TLC plate, and the standard compounds (Table 1) were spotted on to the plate. The next step was incubation of TLC plate in a chamber containing mobile phase as listed in Table 2. The TLC plate was dried after incubation, and sprayed with spraying reagents (Table 3). After the drying of TLC plate, the examination on the color of the spots and their location formed from honey samples were carried out by comparing the resulted spots with those of standard compounds under UV light.

Table 1: Standard compound for identification of secondary metabolites

Type of secondary metabolite	Standard compound
Flavonoid	Quercetin 10mg / 1ml ethanol
Terpenoid	Tymol 10mg / 1ml ethanol
Alkaloid	Quinine 10mg / 1ml ethanol
Phenolic	Gallic acid 10mg / 1ml ethanol
Tannin	Tannin 10mg / 1ml ethanol

Table 2: Mobile phase for identification of secondary metabolite compounds

Type of secondary metabolite	Mobile phase
Flavonoid	Hexane: acetic ethyl: formic acid (6:4:0,2)
Terpenoid	Hexane: acetic ethyl (93:7)
Alkaloid	Toluene: acetic ethyl: diethyl amine (7:2:1)
Phenolic	Acetic ethyl: formic acid: toluene: water (6:1,5:3:0,5)
Tannin	Acetic ethyl formic acid: toluene: water (6:1,5:3:0,5)

Table 3: Spraying reagents for secondary metabolite compounds

Spraying reagent	Type of secondary metabolite	Color
$FeCl_3$	Phenolic	Dark green
$FeCl_3$	Tannin	Green
Anisaldehyde acid	Terpenoid	Purple
Sitroborate	Flavonoid	Yellow
Dragendorf	Alkaloid	Orange

Results and Discussion

Identification of pollen grains extracted from honey samples revealed that the number of pollen types varied among samples from different regencies. The number of pollen types in honey collected from Donggala, Banggai, Morowali, and Parigi Moutong were 33, 36, 30, and 57, respectively. Representatives of pollen types in honey samples were shown in Figure 2. Based on the proportion of each pollen type, all honey samples were classified as multifloral since none of the pollen types being predominant or counted for 45% in their relative number [12].

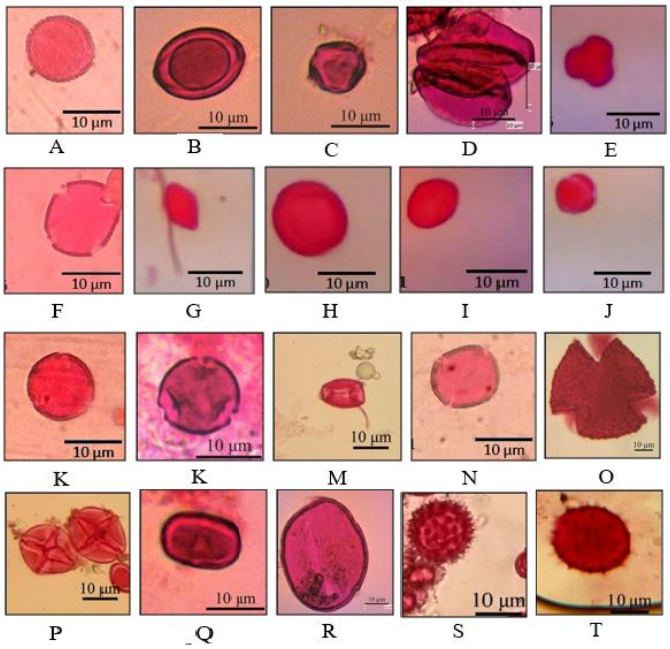

Fig 2: Representatives of pollen types found in honey samples

A. *Aleurites moluccana*, B. *Barringtonia racemosa*, C. *Clausena excavata*,
D. *Cocos nucifera*, E. *Meliosma rigida*, F. *Mussaenda macrophylla*, G. *Pometia pinnata*, H. *Spathodea campanulata*, I. *Styrax formosana*, J. *Vitex rotundifolia*, K. *Urtica dioca*, L. *Canarium* sp., M. *Dioscorea* sp., N. *Impatiens* sp., O. *Linum* sp., P. *Mimosa* sp.,
Q. Apiaceae, R. Arecaceae, S. Asteraceae, T. Malvaceae

Differences in the number of pollen types found in samples from different geographical areas observed in this study indicated differences in plants composing vegetation where honey samples were collected from. The diversity of pollen in honey was known to be highly affected by the vegetation in the area where honey samples were originated [21]. This confirms the role of melissopalynology for the justification of botanical and geographical origin of honey [22]. The certainty of botanical and geographical origin based on melissopalynological research is very important in the authentication of honey and to avoid adulteration [9].
The high diversity of pollen in honey from Parigi Moutong indicated that the area provides suitable habitat and food source for honeybees. Results of this study can be compared with the result of melissopalynological study on honey produced by *A. dorsata* from tropical rainforest in Malaysia by Ibrahim *et al*. [17] since these two areas have similarity in forest type and located in a relatively close geographical region. In terms of pollen content, honey samples from Central Sulawesi showed higher pollen diversity with 30-57 pollen types, compared to the above mentioned study which only found 12 pollen types. Melissopalynological studies on honey produced by *A. dorsata* from India recorded different number of pollen types, such as those from Southern Karnataka with 48 pollen types [16] and from Uttar Pradesh with 37 pollen types [23]. Accordingly, honey samples from Central Sulawesi could be claimed as comparably has high pollen diversity.
Field observations during samples collection revealed that the nests of *A. dorsata binghami* were built on high trees of more than 7 m. The trees include *Mangifera* sp., *Ceiba pentandra*, *Lannea coromandelica*, *Aleurites moluccana*, *Durio* sp., *Arenga pinnata*, *Gliricidia sepium*, and *Ficus* spp. The nests were located on large, primary branches of high trees with a slope of less than 45°. This finding was in line with a study on nesting preferences of *A. dorsata binghami* conducted at Maros forest, South Sulawesi [13]. It is also worth to mention that *A. dorsata binghami* is known to have a specific nesting behavior, which is only one nest found on one tree. Observation on four sampling locations in this study revealed that *A. dorsata binghami* built their nests on trees that grow in the border of dense forest or primary forest and in transition between forest and open areas such as bushes or horticultural plantations. These vegetation types allow the bees to easily fly and roam in their journey for gathering food. Thus the amount and variety of food sources for giant honeybees tend to be high in terms of plant species diversity.
Diversity of pollen types in honey provides information on plant species visited by honeybees during their search for nectar and pollen. Previous melissopalynological studies on honey produced by *A. dorsata* revealed that this species is not selective in visiting plants for feeding [17], and thus forages on various plants including trees, shrub, and herbs [24]. Studies on foraging behavior and plant preferences of *A. dorsata* indicated that this species prefer large flowers with nectar content dominated by sucrose [25]. Meanwhile, an analysis on pollen load collected directly from *A. dorsata* body by Chatterjee [11] found pollen from 14 genera which showed predominate pollen types from plant species located near the hive.
Results of secondary metabolites analysis using TLC showed that all honey samples examined in this study contained 5 classes of secondary metabolite compounds, namely alkaloid, phenolic, flavonoid, tannin, and terpenoid. The data presented in Table 4 showed the number of compounds identified in each class of secondary metabolites, and the Rf value of the spots on the TLC plate. The number of compounds detected in each class of secondary metabolites was expressed as the number of spots (n spots). The Rf (retardation factor) value is the distance traveled by a compound, represented visually as spotted stains on the TLC plate. The Rf value indicates the characteristics of a compound based on its degree of polarity over the mobile phase. Slight differences were found in terms of the number of compounds classified as flavonoid, tannin and phenolic.

Table 4: Secondary metabolites in honey samples from Central Sulawesi

Origin of honey sample	Classes of secondary metabolites									
	Terpenoid		Flavonoid		Tannin		Phenolic		Alkaloid	
	n spot	Rf	n spot	Rf	n spot	Rf	n spot	Rf	n spot	Rf
Donggala	1	0.13	1	0.96	3	0.88 0.91 0.98	1	0.88	1	0.34
Banggai	1	0.13	2	0.63 0.96	3	0.88 0.91 0.98	1	0.88	1	0.34

Morowali	1	0.13	2	0.63 0.96	3	0.88 0.91 0.98	1	0.88	1	0.34
Parigi Moutong	1	0.98	2	0.75 0.85	2	0.85 0.94	2	0.88 0.94	1	0.44

Results of qualitative analysis of secondary metabolites on honey samples from four regencies in Central Sulawesi revealed that they are of good quality, as indicated by the presence of five classes of compounds. A study on multifloral honey produced by *A. dorsata* from Sumbawa showed that the honey contained saponin and flavonoid [26]. Similar studies from other countries emphasized the presence of secondary metabolites in honey produced by *A. dorsata* [27, 28, 29]. It is worth to mention that honey samples from Central Sulawesi has higher number of secondary metabolites compared to those from Sumbawa. This study, therefore, provides important scientific evidence to support the traditional use of honey as health supplement.

Each type of honey has different secondary metabolites content, in which their composition reflected differences in plants visited by honeybees as part of bee's foraging activity [5, 30]. This is due to the fact that secondary metabolites present in honey come from flower nectars collected by honey bees [7]. Kaškonienė and Venskutonis [31] also noted that secondary metabolites content in honey depends on the composition of the pollen or nectar collected by honey bees. Among various secondary metabolites found in honey, phenolic and flavonoid are the most explored for their bioactivity because these two compounds are the main antioxidants in honey [28]. Considering that flavonoids and phenolic are two groups of secondary metabolites most commonly found in honey, Cheung *et al*. [32] underlines that flavonoids and phenolic can be used as botanical markers to identify flower sources and the geographical origin of honey. Nevertheless, Pyrzynska and Biesaga [20] argued that flavonoid and phenolic content of multifloral honey was not only influenced by the flower source and geographical location, but also by the climatic conditions of particular sites where honey was produced.

Physicochemical studies on honey from different geographical origin revealed that secondary metabolites content in honey was affected by types of honey, location of beekeeping, and time of harvest [27]. The same case also applies to the mineral content in honey, which is influenced by the bees, geographical origin, and harvest season [3]. The physicochemical characteristics of honey which vary in samples from different geographical locations is thus potential to be used as the in assessment of honey quality [4], and confirms the use of secondary metabolites content as biochemical marker for authentication of botanical and geographical of honey [12, 33].

Conclusion

Honey produced by *Apis dorsata binghami* from Central Sulawesi has high pollen diversity, and classified as multifloral honey. Qualitative analysis of secondary metabolites using TCL method showed that the honey has 5 classes of compounds, namely alkaloid, phenolic, flavonoid, tannin, and terpenoid. Based on the analysis of pollen diversity and secondary metabolites content, all honey samples could be categorized as high quality. Accordingly, this study contributes in providing scientific basis for promoting the use of honey as a food supplement of health benefit.

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