

Bioactive Contents, BSLT Toxicity and Antioxidants from *Apis dorsata* Binghami and *Apis mellifera* Nest Extracts

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Abstract

Beenest are rich in secondary metabolites because honeycombs, among others, are formed from plant resins (propolis). This study aimed to analyze differences in flavonoid content and in vitro antioxidant activity of *Apis dorsata* Binghami and *Apis mellifera* nest extracts. The samples used were *A. dorsata* from North Sulawesi and *A. mellifera* from South Sulawesi. The beenest was extracted using 95% ethanol solvent. Beenest extract was analyzed for its flavonoid content by the HPLC method, toxicity was tested by the BSLT method, and in vitro antioxidant activity was tested by the DPPH method. The results of the analysis of flavonoids showed that *A. dorsata* nest extract produced 21 types of compounds while *A. mellifera* produced 26 types of compounds. The toxicity test results showed that the *A. dorsata* nest extract had a better LC_{50} of 245,69 mg/l than *A. mellifera* nest extract with an LC_{50} of 443,70 mg/l. The results of the in vitro antioxidant test of *A. dorsata* nest extract were stronger, namely IC_{50} 1.16 mg/l, compared to *A. mellifera* nest extract IC_{50} 2.40 mg/l, although both were included in the category of powerful antioxidants. In vitro, anticancer test results on MCF-7 cells, *A. mellifera* nest extract was active with IC_{50} 100.02mg/l. Compared to *A. dorsata* extract, it was active with IC_{50} 102.22mg/l, but the two were not significantly different. Based on the analysis of flavonoid content, toxicity test, and antioxidant test, *A.dorsata* and *A.mellifera* beenest extracts have potential as in vitro antioxidants

Keywords: *Apis dorsata* Binghami, *Apis mellifera*, Bioaktif, Antioxidant



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INTRODUCTION

Indonesia has the second-highest animal and plant biodiversity in the world. More than seven species of honey bees are endemic to Indonesia. *Apis dorsata* Binghami is an endemic honeybee species found on the island of Sulawesi (Mocosuli et al., 2019).

Honey bees are part of a class of insects that benefit humans and play an important ecological role. Since prehistoric times, humans have used the secondary metabolites of bees, namely honey, propolis and toxins as food and medicinal ingredients. As pollinating organisms, each year bee species pollinate more than 70% of flowering plants and up to 6.1 billion agricultural dollars produce products from bee pollination (Raffiudin, 2002; Mocosuli, 2013).

Based on the shape of the nest, honey bees can be divided into two: honey bees with open nesting and honey bees with cavity nesting (Mayr et al., 2020). *Apis dorsata* is a honey bee with an open nest with only one comb. *Apis mellifera*, or the western honey bee, has nests in cavities with more than one comb (Mocosuli, 2013; Hadissoesilo, 2007). *A. dorsata* is the most productive in producing honey which has a nest with only one large comb, which usually hangs from tree branches, open ceilings, and cliffs or rock ravines. The giant forest honey bee (*A. dorsata*) is one of the endemic species of Sulawesi; currently, *A. dorsata* has not been cultivated and still lives naturally in the forests of Sulawesi (Mocosuli, 2013). In contrast, *A. mellifera* has been cultivated for a long time by humans. *A. mellifera* can live and develop well in artificial nests made of wooden boxes (Cunningham et al., 2022). The diversity of food sources for *A. mellifera* is less than that of *A. dorsata*.

Research on bioactive *A. dorsata* has been started by researchers since 2013 for anticancer, antibacterial, antihyperlipidemic, and antioxidant activities (Mocosuli, 2013; Mocosuli et al., 2019). Honeycomb is rich in secondary metabolites because honeycomb, among others, is formed from plant resin (propolis) (Dvykaliuk et al., 2022). Plant propolis, used as the main ingredient in forming beehives, generally comes from more than one plant species (Durazzo et al., 2021). However, *A. dorsata* nests have a higher diversity of plant sources than *A. mellifera*. The secondary metabolites of the hive and the honey produced by each honey bee species are strongly influenced by climate and plant diversity around the hive (Rasyiid & Susandarini, 2020).

Propolis is a natural resinous mixture that bees collect from various plant species. Bee propolis is the product of processed plant resins with bee saliva, which contains various enzymes and peptides to produce new resins (Kieliszek et al., 2023). Honeybees collected plant resins from plant parts, shoots, and exudates (Wagh et al., 2013; Abdullah et al., 2020). Propolis also has a high micronutrient content such as vitamins (A, B, C), minerals (Ca, Cu, Fe, Mg, Mn, Na, Fe, and Zn), and the enzyme succinate dehydrogenase. The most abundant secondary metabolites contained in propolis are polyphenols (phenolic acids, flavonoids, and their esters), steroids, terpenoids, and amino acids (Bei-Srairi et al., 2020; Sforcin & Bankova, 2011). Propolis has a color and bioactive content that varies depending on the plant source (Sforcin & Bankova, 2011; Abdullah et al., 2020). The natural resinous substance in propolis gives it a sticky aroma and texture at the temperature of the newly formed nest. The health benefits of propolis include accelerating cell regeneration, repairing leaky blood vessels, curing diabetes mellitus, stimulator of the immune system, anticancer, antiprostaglandin, anti-stress, antioxidant, anti-inflammatory, antibacterial, antihyperlipidemic and anticancer (Hegazi 1998; Bankova, 2000; Finstrom and Spivak, 2010; Kubiliene, et. al. 2015; Kumazawa et. al. 2004). Propolis in beehives has excellent potential as an antiviral agent for SARS-COV2 (Baretta et al.,

2020). Honeycomb propolis contains antioxidant and anticancer flavonoids (Dogan et al., 2020).

Propolis extract contains compounds belonging to flavonoids, phenols, triterpenoids, mono- and cisquiterpenes, tannins and alkaloids. Flavonoids are one of the most important constituents, representing about 50% of propolis content. The composition of propolis is very complex and mainly contains waxes, resins, and volatiles. The main chemical groups present in propolis resin consist of phenolic acids or their esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols and chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenda and b-steroids (Tsuda & Kumasawa, 2021). Flavonoid content depends on the area where the propolis is collected because plants, geographical factors, and weather influence its characteristics. Flavonoid compounds are compounds that dissolve in water and have antioxidant activity. Based on this background, research was carried out on the bioactive content, toxicity of BSLT, and antioxidants of *A. dorsata* and *A. mellifera* nest extracts.

METHOD

Beenest samples of *Apis dorsata* and *Apis mellifera*

This research was conducted from July 2022 to January 2023. *A. dorsata* and *A. mellifera* nests were extracted at the Laboratory of Bioactivity and Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Manado State University, and Basic Laboratory of Sam Ratulangi University, Manado. Analysis of the flavonoid content by the HPLC method, the toxicity test by the Brine Shrimp Lethality Test (BSLT) method, and the in vitro antioxidant test were carried out at the Laboratory of the Center for Biopharmaca Studies IPB Bogor. Nest samples of *A. dorsata* were obtained from Ratahan forest, Southeast Minahasa, North Sulawesi. Samples of *A. mellifera* nests were obtained from beekeeping in Bokin Village, Rantebua Toraja Utara district, South Sulawesi. Beehives are cleaned of dirt before being extracted. The beehives used for extraction are nests not occupied or found with eggs or bee larvae.

Research Methods

This research was conducted in several stages consisting of the first stage of extracting the nests of *A. dorsata* Binghami and *A. mellifera*, the second stage of flavonoid analysis, the third stage of the BSLT toxicity test, the fourth stage of in vitro antioxidant activity test using the 1,1 – diphenyl – picrihidazyl (DPPH) method.

Simplisia preparation

The samples used were *A. dorsata* and *A. mellifera* nests. Samples are cleaned and dried to avoid bacteria and fungi that can cause decay.

Extraction

Extraction was carried out using the Maceration method (Harborne, 1996). Fresh nests of *A. dorsata* and *A. mellifera* were weighed and mashed using a blender, 250 grams, and 100 grams, respectively. Each was poured into a jar containing 1,000

ml and 400 mL 96% ethanol (1:4 ratio) for 3 x 24 hours at room temperature, every 6 hours shaken using an orbital shaker for 30 minutes. The filtrate was filtered using Dr. Watts filter paper. The extracted filtrate was then used as a solvent using a Heidolph rotary evaporator at 54°C and 55 rpm. The resulting solvent evaporation is honeycomb ethanol extract (EESL) of *A. dorsata* and *A. mellifera*.

Compound Content Analysis

The EESL *A. dorsata* and EESL *A. mellifera* flavonoids were analyzed using High Performance Liquid Chromatography (HPLC). Each of the two grams of EESL sample was added with 14 mL of 70% acetonitrile, then left for 24 hours. Samples were filtered using filter paper (Whatman No.41) and Polyvinylidene fluoride (PVDF) filters (Millipore). Measurement of flavonoid levels was carried out using HPLC (Mokosuli et al., 2019).

Toxicity test against *Artemia salina* Leach BSLT method

Hatching cysts of *A. salina* Leach. *A. salina* cysts were weighed as much as 50 mg and then put into a container containing filtered seawater; after aeration, the cysts were left for 48 hours under lamplight so that they hatched completely. The hatched larvae are taken to be used in the toxicity test. Toxicity test against *A. salina*. The toxicity test was carried out separately according to the type of beenest extract. The working stages of the toxicity test were as follows: ten *A. salina* larvae were put into a vial containing seawater, and then added a solution of honeycomb extract so that the final concentration was 1000 ppm, 100 ppm, and 10 ppm. Observations were made after 24 hours by counting the number of dead larvae from the total larvae included in the vials. Calculations using the help of a magnifying glass. According (Mokosuli, 2021) the cumulative percent mortality data processing used LC₅₀ probit analysis with a 95% confidence interval in the IBM SPSS 20 program.

DPPH Assay

Beenest ethanol extract (EESL) was prepared in various concentrations (10, 50, 100, 200, and 250 ppm). Each was put into a test tube. Into each test tube, 500 µl of 1mm DPPH solution in methanol was added. The volume was made up to 5.0 ml, then incubated at 37°C for 30 minutes; then, the absorbance was measured at a wavelength of 515 nm. As a positive control and for comparison, vitamin C (2, 3, 4, and 5 ppm concentration) and Butylated hydroxytoluene (BHT) (2, 4, 6, and 8 ppm concentration) were used. The IC₅₀ value is calculated respectively using the regression equation formula (Wołosiak et al., 2021; Samuel & Rombot, 2023).

$$\% \text{ Scavenging} = \frac{([\text{DPPH Absorbance} - \text{The absorbance of the test sample}])}{([\text{DPPH Absorbance}])} \times 100 \%$$

Data Analysis

Data from the HPLC method of flavonoid analysis were analyzed descriptively. The toxicity of the BSLT method extract. The LC₅₀ value is the concentration (ppm) needed to kill 50% of *A. salina* Leach shrimp larvae. The

cumulative percent mortality data processing used LC_{50} probit analysis with 95% confidence interval in IBM SPSS 20 program. Antioxidant activity. The IC_{50} value is the extract concentration required to scavenge 50% DPPH radicals.

RESULT AND DISCUSSION

Extraction

Nest samples of *A. dorsata* were obtained directly from natural nests in the Ratahan forest, Southeast Minahasa. Samples of *A. mellifera* were obtained from beekeeping in Bokin Village, Rantebua Toraja Utara District, South Sulawesi. The nest sample of *A. dorsata* is golden yellow, the well where the eggs are laid is hexagonal but larger than *A. mellifera*. The nest sample of *A. mellifera* is pale yellow, the hole where the eggs are laid is smaller. *A. dorsata* and *A. mellifera* nests were harvested fresh and preserved in sample boxes from locations with an average temperature of 25° C (Figure 1).

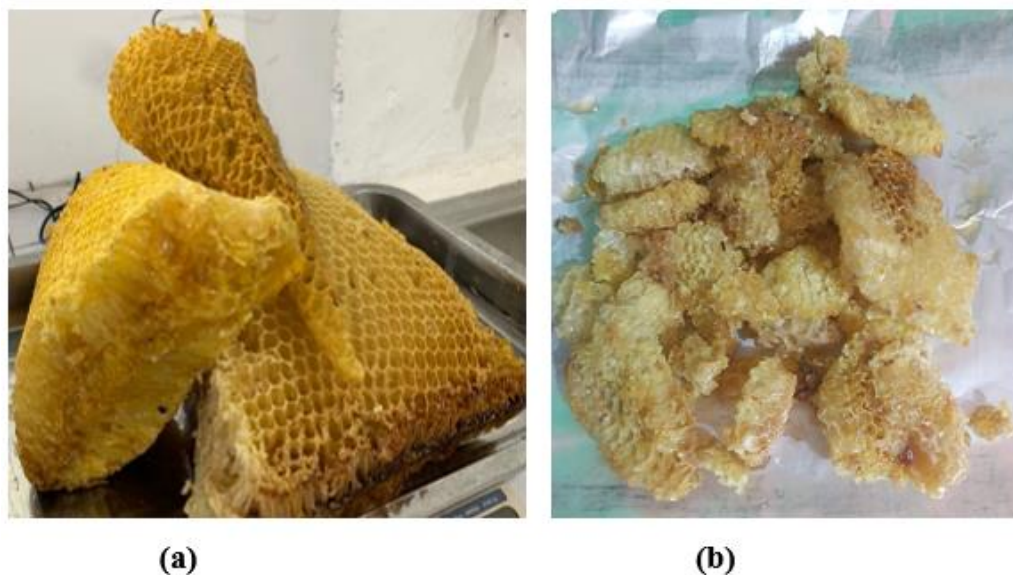


Figure 1. Bee Nest Sample (a). *Apis dorsata* Binghami (b). *Apis mellifera*

Fresh samples were prepared before being extracted by the maceration method. The nest sample used is a nested sample where the hexagonal well where the eggs are not filled with bee eggs. The sample is mashed to increase the surface area when it is macerated with a solvent. The extraction was carried out with 95% ethanol solvent aiming at the efficiency of withdrawing bioactive from hive simplicia. The comparison of simplicia and solvent 1:4 (w/v) is based on previous research, where the extraction efficiency of *A. dorsata* nests was obtained at a ratio of 1:4 and 95% ethanol solvent (Mokosuli et.al. 2019). The filtrate of *A. dorsata* was blackish brown while the filtrate of *A. mellifera* was light yellow. The aroma of *A. dorsata* nest extract has a sharper honey smell compared to *A. mellifera*. After the solvent was evaporated, the crude extract of *A. dorsata* Binghami was dark brown, while the crude extract of *A. mellifera* was yellow (Figure 2).

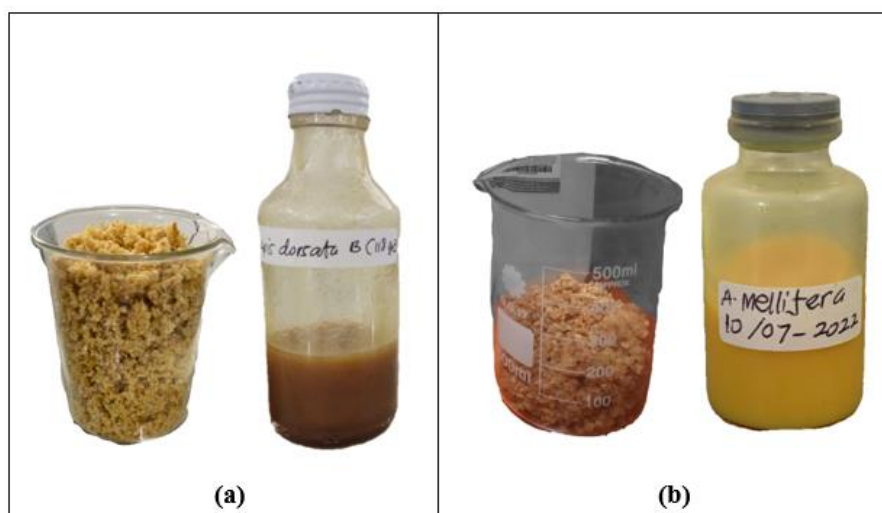


Figure 2. Simplisia and extract (a). *Apis dorsata* Binghami (b). *Apis mellifera*

The yield percentage of *A. mellifera* nest extract was 60%, while that of *A. dorsata* was 23.6%. The yield percentage of *A. mellifera* was greater than that of *A. dorsata* (Table 1). Yield is the ratio of the product's dry weight to the raw material's weight. Although the weights of *A. mellifera* and *A. dorsata* simplicia used differed, the solvent and simplicia ratios were the same. The difference in the amount of simplicia is due to the availability of simplicia. Simplisia *A. mellifera* was obtained from South Sulawesi, while Simplisia *A. dorsata* was obtained from Minahasa. The nests of *A. dorsata* are more significant than those of *A. mellifera*. Extract yield was calculated based on the ratio of the final weight (weight of the extract produced) to the initial weight (weight of the cell biomass used) multiplied by 100% (Sani et al., 2014). The yield value is also related to the extract's bioactive content. Based on the yield percentage obtained, it is assumed that the bioactive content of *A. mellifera* is more than *A. dorsata*. Ethanol is a polar solvent; therefore, in theory, it is a polar bioactive compound that will significantly interest the extract (Harborne, 1996).

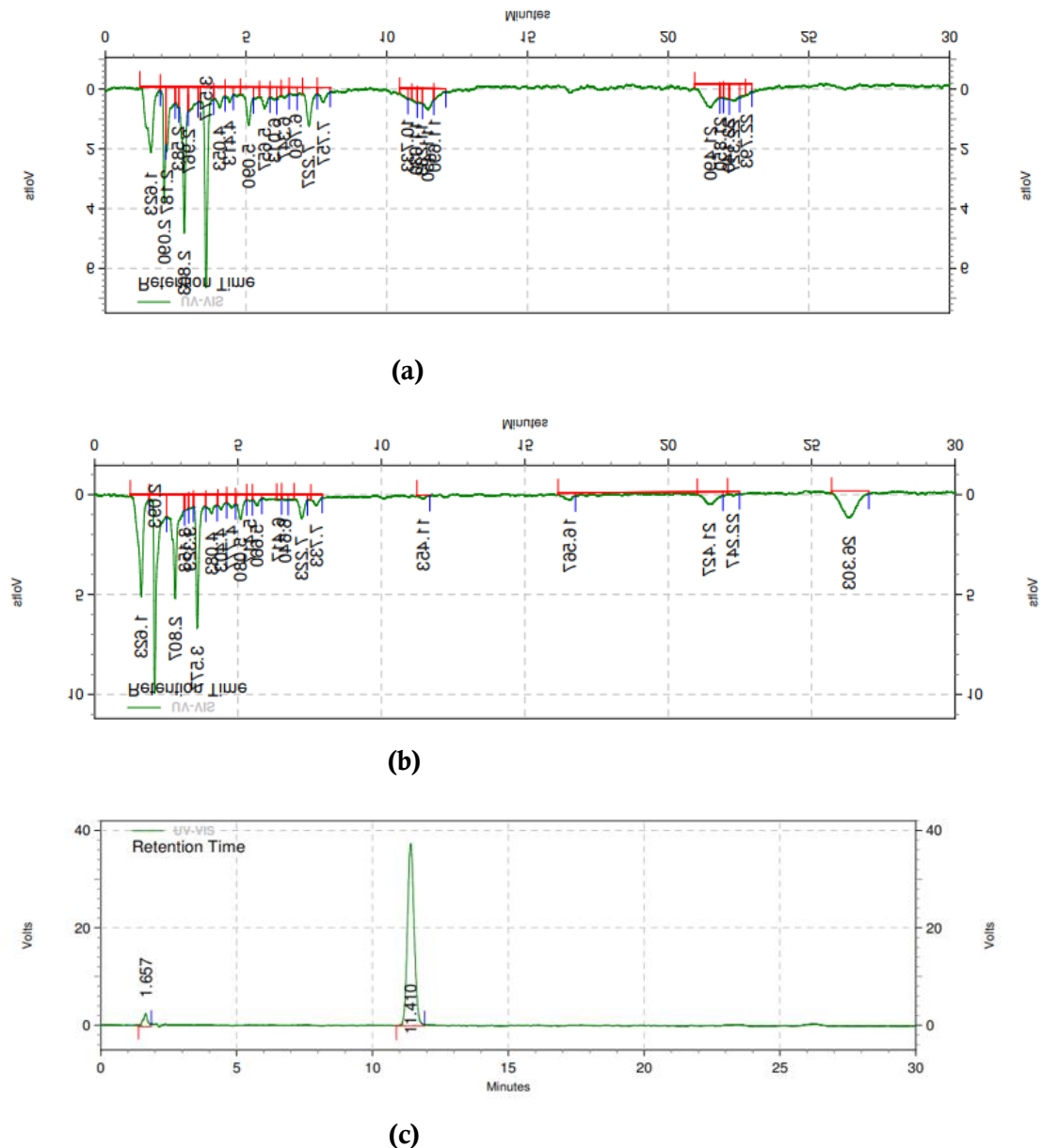
Table 1. Yield Value

Simplisia	Ethanol Solvent Volume 96% (mL)	Simplisia Weight (g)	Extract Weight (g)	Yield (%)
<i>A. mellifera</i> nest	400	100	60	60
<i>A. dorsata</i> Binghami nest	2000	500	118	23,6

The sample component compounds require retention time to pass through the column to the detector. The retention time is calculated from when the sample is injected to the peak of the maximum reading on the detector. Different compounds will have different times so that each concentration can be calculated. The chromatogram describes the sample components seen from the number of peaks (peaks) produced. The concentration of the component to be analyzed can be calculated by comparing the peak area of the component with the peak area and standard concentration. Based on the number of peaks produced and the retention

time, *A. dorsata* produced 21 types of compounds, while *A. mellifera* produced 26 types of compounds.

Based on the retention time, both *A. mellifera* nest extract and *A. dorsata* nest extract contained quercetin. Quercetin standard retention time is 11.410. The *A. dorsata* extract obtained a retention time of 11,453. In the *A. mellifera* extract, four peak retention times were obtained, close to quercetin, namely 11,020, 11,233, 11,450, and 11,690 (Figure 3). Thus it is suspected that four types of quercetin derivatives are detected in *A. mellifera* extract.



Toxicity test against *Artemia salina* Leach BSLT method

The Brine Shrimp Lethality Test is an initial pharmacological screening commonly used to determine the bioactive potential of a plant extract. *Artemia salina* leach fry have a cell-like structure but are an individual or organism. *Artemia salina* leach larvae have a thin membrane that allows the diffusion of the test extract solution into the body. Based on the BSLT test results, the mortality percentage increased or was linear with increasing test concentration. However, the highest mortality percentage was at the 1000000 µg/ml test concentration 100% *A. dorsata* nest extract compared to 90% *A. mellifera* nest extract (Table 2).

Tabel 2. Mortalitas Larva *A. salina* Leach

<i>Apis dorsata</i>							
Concentration (µg/ml)	Number of individuals	Replication			Total	Average of mortality	% mortality
		1	2	3			
1000000	10	10	10	10	30	1.00	100.00
500000	10	8	7	8	23	0.77	76.67
100000	10	5	4	3	12	0.40	40.00
50000	10	2	1	3	6	0.20	20.00
<i>Apis mellifera</i>							
Concentration (µg/ml)	Number of Individuals	Replication			Total	Average of Mortality	% mortality
		1	2	3			
1000000	10	9	8	10	27	0.90	90.00
500000	10	8	6	5	19	0.63	63.33
100000	10	2	3	3	8	0.27	26.67
50000	10	1	0	0	1	0.03	3.33

Based on the mortality data of *A. salina* larvae, a probit analysis was performed to determine the LC₅₀. The results of probit analysis showed that the LC₅₀ of *A. dorsata* nest extract was higher than that of *A. mellifera* nest extract (Figure 5).

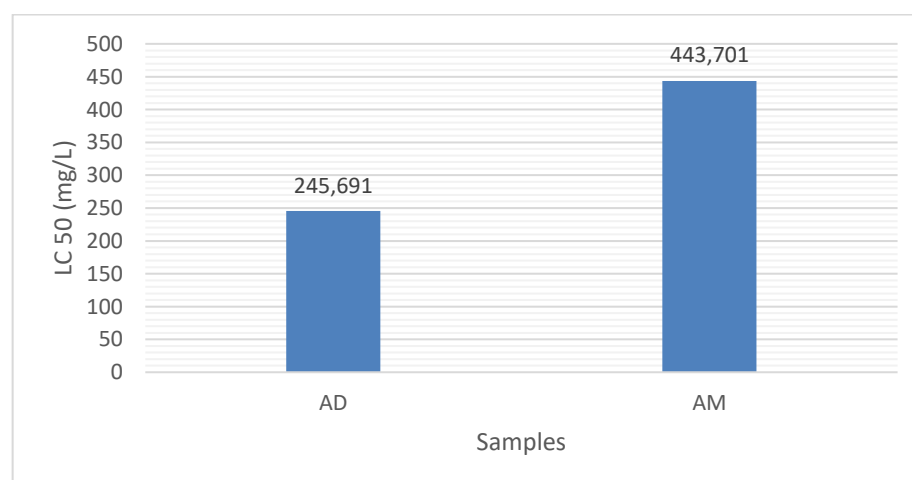


Figure 4. Results of probit LC₅₀ analysis of *A. dorsata* nest extract (AD) and *A. mellifera* nest extract (AM)

The DPPH method antioxidant test measures the scavenging power of 1,1 – diphenyl – picrihidazyl free radicals. DPPH is a stable free radical compound, so when used as a reagent in free radical scavenging tests, it is sufficiently dissolved when stored in a dry state under good and stable storage conditions for years. DPPH free radical scavenging is based on reducing the colored DPPH methanol solution by free radical inhibition. Based on the research, the results of the DPPH free radical inhibition test by *A. dorsata* and *A. mellifera* nest extracts were after incubation for 30 minutes, and the absorbance was measured at a wavelength of 512 nm.

Ten test concentrations and three replicates of *A. dorsata* and *A. mellifera* nest extracts were used to obtain the absorbance of the test solution against DPPH. The absorbance data for each concentration is then used to determine the percentage of inhibition. Based on the inhibition percentage and test concentration, the regression equation for the antioxidant activity of *A. dorsata* nest extract was $Y = 0.361 + 0.016 x$ (Figure 6). Based on the inhibition percentage and test concentration, the regression equation for the antioxidant activity of *A. mellifera* nest extract was $Y = 0.454 + 0.039 x$.

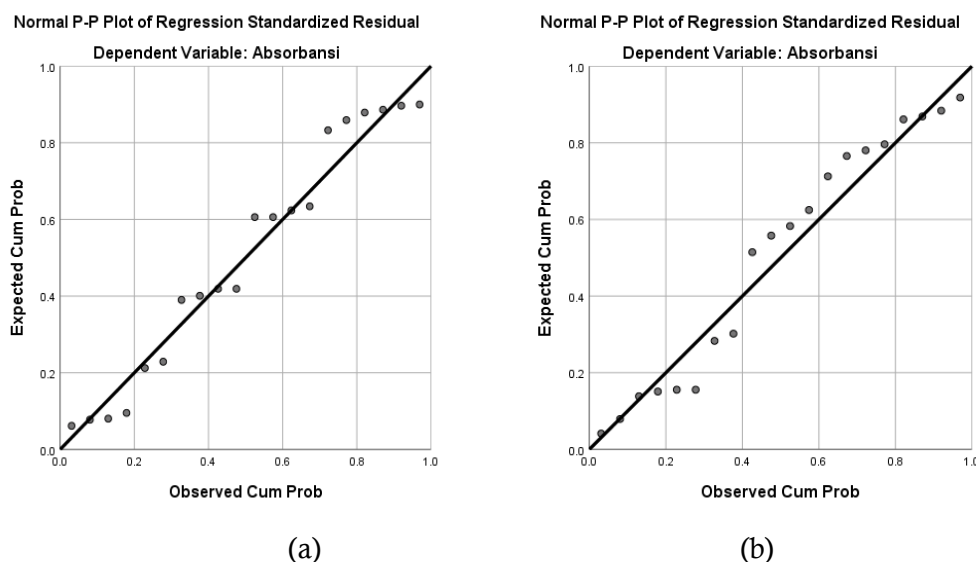


Figure 6. Regression Analysis curve of antioxidant activity: (a) *A. dorsata* extract (b) *A. mellifera* extract

From the results of the regression analysis, it was found that the IC 50 of the *A. dorsata* nest extract was 1.161 mg/l while that of the *A. mellifera* nest extract was 2.404 mg/l (Figure 7). Thus the nest extract of *A. dorsata* showed better scavenging of DPPH free radicals than *A. mellifera*.

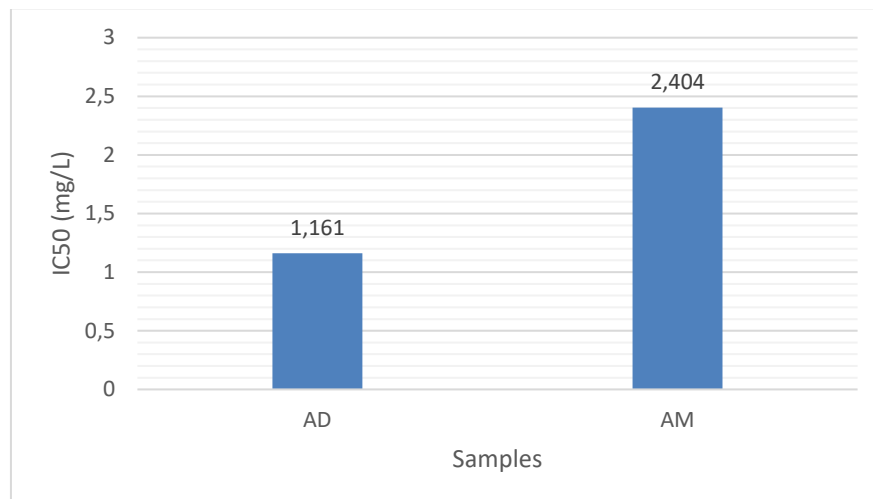


Figure 7. Results of probit IC 50 analysis of *A. dorsata* hive extract (AD) and *A. mellifera* nest extract (AM)

DISCUSSION

Bioactive extraction and analysis

Extraction of *A. dorsata* (AD) and *A. mellifera* (AM) nests was successfully carried out using 95% ethanol. The highest yield was shown by AM extract compared to AD because AM simplicia had less water content than AD simplicia. Fresh nest of *A. dorsata* contains more water than fresh nest of *A. mellifera*. *A. dorsata* bees are bees with open nesting while *A. mellifera* has cavity nesting, this causes AD hives to contain more water than AM hives (Mokosuli & Rombot, 2022; Mokosuli et al. 2021). Honey beehive extraction with ethanol will produce more than 90% propolis. Therefore honeycomb extract with ethanol is often called propolis ethanol extract.

The results of the analysis of the flavonoid content of the AM extract had a higher variance of flavonoids than the AD extract. The yield presentation shows the amount of bioactive content in the extract (Mangiring et al., 2018; Wen et al., 2018; Sood & Gupta, 2015). The yield of AM is higher than that of AD, so the bioactive content is more than AD. Flavonoids have been reported as the highest secondary metabolite content in honeycomb extracts. Apart from flavonoids, many phenolic compounds are contained in honeycomb and propolis extracts (Asem et al., 2020; Mokosuli & Rombot, 2023; Bouchelaghem et al., 2022; Mokosuli et al., 2019; Jamil and Zhari et al. 2019; Mouhoubi et al., 2016). Quercetin is a group of flavonoids most abundant in propolis obtained using ethanol (Wang, 2016; Najafpour et al., 2019). However, the geographical location of the hive, the season, and the bee-feed plants significantly affect the secondary metabolite content of propolis (Mokosuli et al., 2023; Kurek et al., 2022; Alvear et al., 2021; Sarikahya et al., 2021).

According to Thamrin et al., (2016), a compound is said to be a powerful antioxidant if the IC₅₀ value is ≤ 50 ppm and a compound is said to be a potent antioxidant if the IC₅₀ value is between 50-100 ppm, a compound is said to be a moderate antioxidant if the IC₅₀ value is between 101-150 and the compound is said to be weak antioxidant if the IC₅₀ value is more than 151 ppm. Based on the IC₅₀ values obtained through the linear regression equation, it can be concluded that the

extracts of *A. dorsata* and *A. mellifera* nests are included in the very strong t category ($IC_{50} \leq 50$ ppm). This is due to the high content of flavonoids.

The DPPH free radical scavenging activity by AD and AM extracts was due to the high content of flavonoids. The antioxidant activity of AD extract is stronger than that of AM extract. This shows that the number of flavonoid variants does not determine the antioxidant activity. Certain classes of flavonoids have strong antioxidant activity. Both AD and AM extracts showed the content of several quercetin derivatives. It is suspected that quercetin has strong DPPH-free radical scavenging activity. The large number of hydroxy groups (OH) in flavonoids causes strong free radical scavenging activity. Flavonoid aglycones with ring B without a hydroxyl group (OH) or with OH groups in rings A, B, and C blocked by a methyl group (-CH₃) or an ester group (-COR). It is known that the free OH group at this position suggests an important role in the stabilization of the flavonoid molecule after a “free radical attack” (Okinczyc et al., 2021). The unpaired electron in DPPH gives a strong absorption, maximum at $\lambda = 517$ nm, and is colored purple (Figure 8). Free radical scavenging by antioxidants occurs when unpaired electrons become paired in the presence of a hydrogen donor, thus forming a more stable DPPH (Syed et al., 2021).

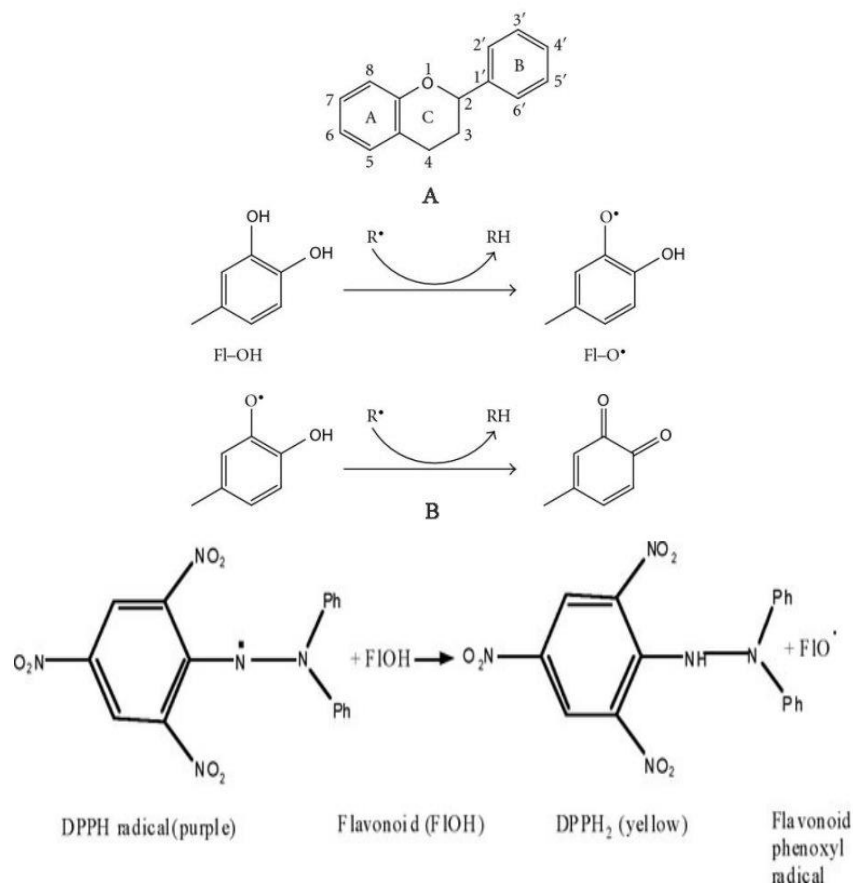


Figure 8. Free Radical Attenuation by Flavonoids. (a) The basic structure of flavonoids. (b) Free Radical Suppression Process by Flavonoids

CONCLUSION

Based on the research results, it can be concluded: 1). *A. mellifera* nest extract contains a higher variant of flavonoids than *A. dorsata* . 2). In vitro antioxidant activity of *A. dorsata* and *A. mellifera* nest extracts is very strong, although based on IC₅₀, *A. dorsata* nest extracts are stronger than *A. mellifera*.

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