Potential antioxidant and anticancer effect of Apis dorsata Binghami Crude Venom from Minahasa, North Sulawesi

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Abstract
This current research aims to find the characteristics and composition of the crude venom Apis dorsata Binghami, its activity of free radical DPPH scavenging, and cytotoxic activity. The analysis of honey bee venom peptide composition was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Phytochemical contents were analyzed using Harborne method, antioxidant activity was measured using DPPH free radical scavenging method and the anticancer activity in vitro was analyzed for cytotoxic effects on cancerous cells of murine leukemia p388 cell line. The results showed that bee venom AD1 has 5 bands of molecules with a molecular weight of 33,53 kDa; 32,21 kDa; 21,51 kDa; 6.1 kDa and 2.67 kDa; AD2 has molecules of 33,52 kDa; 21,51 kDa; 14,43 kDa; 6,14 kDa and 2.43 kDa. Apis dorsata crude venom of Minahasa in North Sulawesi contains hyaluronidase/phospholipase A; phospholipase; lysophospholipase or protease inhibitor 5 antigens, and melitin. An unknown tupe of peptide was also identified on fourth band. The analysis of phytochemicals revealed that the crude venom also contains flavonoids and polyphenols. AD1 antioxidant activity was present (IC₅₀:103,28 ppm), as well as the AD2 (IC₅₀:139,13 ppm) compared to control BHT (IC₅₀:142,38 ppm). Cytotoxic activity was observed in murine leukemia p388 cells by AD1 (IC₅₀:36,12 µg/ml), AD2 (IC₅₀:32,21 µg/ml) and positive control Canamycin (IC₅₀:42,06 µg/ml). Thus, Apis dorsata Binghami crude venom from Minahasa, North Sulawesi is potential to be developed as a source of bioactive antioxidant and anticancer.

Keywords: Crude bee venom, Apis dorsata Binghami, radical scavenging, cytotoxic, Minahasa

1. Introduction
Apis dorsata Binghami is Sulawesi endemic honey bees, known for its giant honey bees (Hadisoesilo, 1997; Raffiudin, 2002) [12]. Until currently, A. dorsata Binghami has received little scientific attention compared to its closest relative, Apis mellifera. In addition to producing honey, propolis and bee pollen, honey bees also produce bee venom (BV). The biochemical composition of BV is very unique and has been an interesting research topic to date. Overall bee venom of bees consists of around 120 active chemical components, with the components of the 40s is already detected, including 11 peptides, 5 enzymes, 3 amine, carbohydrates, fat and amino acids. Many of the current research reports found that the most peptides that play a role in bee venom is melittin, apamin, mast cell degranulating peptide (MCDP) and adolapin (NCBI, 2010) [31]. Honeybee venom is a complex mixture between the low molecular weight polypeptide enzymes. A number of enzymes that was reported to be contained in honey bee venom include phospholipase A2, hyaluronidase, phosphomonooesterase acid esterase, α-D-glucosidase, lysophospholipase, α-galactosidase and α-aseltaminodioesiguinosidase, and arylamidase (Hassanein and Hegab, 2010) [14]. Until now, there has been little research about the bioactivity of A. dorsata Binghami venom in the field of pharmacology. It was stated that bee venom is a form of evolution that takes place among bees which caused this species occupies almost every area on planet earth. This has led to the study of the bioactivity of bee venom into an interesting and broad research field of study (Zalat et al., 2002) [39]. In our preliminary study, we found that the composition of the honey bee venom in Minahasa influenced by season, habitat and food source within radius of 700-1000 meters from the natural nesting site (Mokosuli, 2013) [28, 44]. Therefore the analysis of the bioactive contents of crude venom is very important in an effort as can be used as raw material for bio-pharmacy.
Food source research, we have found these types of plants are the main source for establishment and development of the bees. In other regions, many endemic plants species as a source of pollen and nectar. North Sulawesi, more specifically the Minahasa region, has many endemic plants species as a source of pollen and nectar. This area is known as the habitat of bees. In the Minahasa region, the composition of nectar and pollen that was formed and subsequently became the ingredients of honey bees feed. (Heldt and Heldt, 2005) [15]. Therefore, the composition of secondary metabolites including process and characteristics of secondary metabolites, produced honey bee namely honey, propolis, wax, venom are also heavily influenced by the kind of available plants in its natural habitat. Bees that live in one area will have a different component of secondary metabolites. Hadisoesilo (1997) [12] states that a worker bees, Apis cerana and Apis nigrocincta living in Sulawesi have distinct foraging activity and different types of plants. Previous studies conducted by the researchers found the antibacterial activity, antioxidant and anticancer of bee venom (Chmielewska and Szczesna, 2004; Mokosuli, 2013) [5, 28, 44].

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Cancer is a disease that results from disruption of cellular metabolism mainly due to damage of genetic materials that exist within the cell (McKelvey and Evans, 2003) [25]. Bee venom can serve as a modulator of rheumatoid arthritis that arise from radical oxygen species (ROS) activity (Murakami, et al., 1997; Oren and Shai, 1997; Hassanein and Hegab, 2010) [26, 12, 14]. BV has been known to induce apoptosis in many types of cancer cell culture (Reference?). BV induces morphological changes and a decrease in the percentage of viable cells in a culture of cervical cancer cells. Flow cytometric analysis showed that BV induces the production of ROS, improving the content of cytoplasmic Ca2+; reduces mitochondrial membrane potential by releasing cytochrome oxydase, and promoting the activation of caspase-3 that induce apoptosis. BV also induces an increase in the tumor suppressor gene p53, Fas, p21 and Bax, but lowers oncogenes and Bcl-2 (Wan et al., 2008) [38]. BV significantly inhibits the growth of cancer cells in the lungs (Reference?). BV honey bees also inhibits vascular endotheloal growth factor (VEGF) that induces cell proliferation (Huha, et al., 2010) [16]. This current research aims to analyze the venom composition A. dorsata Binghami from Minahasa, North Sulawesi, its free radical scavenging activity (antioxidant) and cytotoxic activity on cancer cells in vitro.

2. Materials and Methods
2.1 Samples Collection: A. dorsata obtained from from Kombi Forest (Minahasa) and Kaweruan Forest (North Minahasa) (Fig 1). As many as 200 individual A. dorsata Binghami were collected from a natural nesting by using net. Living A. dorsata Binghami from each location were transported to the Molecular Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Manado. Isolation of bee venom was carried out by accommodating the liquid venom in eppendorf vial and stored at temperature 0°C before use.

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**Fig 1:** Location A. dorsata Binghami sampling in natural nesting, (A) the forest of Kaweruan and (B) forest of Kombi.
2.2 Materials: The materials used include: acrylamid (Merck), ddH2O, bisacrylamid, ammonium peroxodisulphate, a protein marker 4.6-100 kDal (Merck), TEMED, ethanol p.a. (Merck), aquadest., 1-diphenyl-2-picirillylhydrazil (DPPH) (Merck), butyl hydroxy toluene (BHT), methanol (Merck) pa. Murine P388 leukemia cancer cells were obtained from the Laboratory of Chemistry of Natural Materials, Institute of Technology Bandung (ITB) Indonesia, media Rosewell Park Memorial Institute (RPMI) 1640, fetal bovine serum, canamycin, dye reagent [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], a solution of 10% SDS-0.01 N HCL. Tools used 1 set of electrophoresis vertical model TV100YK-MODSYS, a set micropipette Eppendorf, centrifuge Eppendorf, UV-Vis Spectrophotometer Perkin Elmer Lamda 35, ultracentrifuse Eppendorf 5430R, Waterbath mammert, incubator mammert, digital scales, hot plate, nanospectrophotometer, microplate reader, CO2 incubator, glasses and other devices.

2.3 SDS PAGE: SDS-PAGE using a modification of the method of Laemmli (1970) [24] with poliacrylamid concentrations of 17.5% was performed. The composition of the gel separator prof 17.5% while collecting gel composition Prof. 5%. After the gel was created, the sample and the protein marker were injected to existing wells. Running was performed for 4 hours on a 60 volt, 20 mA. Protein gel would sink with the help of an electric current that moves from the negative to the positive pole. After running, the gel was passed on to the silver staining. The gel was then soaked in a solution of fixation for approx. 2 hours, while being agitated slowly. The gel was then washed with a solution of washing solution for 20 minutes (repeated 3 times) without agitation. Gel was rinsed with free-ion aquadest (ddH2O) for 10 seconds. The gel is then soaked in a solution of sensitize for 1 minute. Gel washed again in ddH2O for 20 seconds (repeated 3times). Gel was then incubated into the fridge with AgNO3 0.1% (silver nitrate) for 20 minutes and washed or soaked with ddH2O for 20 seconds and repeated 3 times. Gel was then soaked with a developing solution, while agitated up to a staining dye. Gel was added with a stop solution for 5 minutes then rinsed with ddH2O for 5 minutes. Scanning was done and subsequent data analyses were performed.

2.4 Antioxidant test: Crude venom bee was made in the distribution of concentrations of 10, 50, 100, 200 and 800 ppm. Each was put into test tubes. Each of the test tubes was added with 500 µl DPPH solution 1 mm in methanol. Subsequent data analyses were performed. The antioxidant activity IC50 value is concentration required to scavenge DPPH free radical by 50%. The data were analyzed by linear regression equations using SPSS 20 (IBM). The antioxidant activity IC50 value is concentration of the extract that is required for the inhibition of growth of murine P388 leukemia cancer cells by 50%. The data were analyzed by linear regression equations using SPSS 20 (IBM).

2.5 Data Analysis Techniques: Isolation and characterization of the results of bee venom were analyzed qualitatively. The antioxidant activity IC50 value is concentration required to scavenge DPPH free radical by 50%. The data were analyzed by linear regression equations using SPSS 20 (IBM). The anticancer activity IC50 value is the concentration of the extract that is required for the inhibition of growth of murine P388 leukemia cancer cells by 50%. The data were analyzed by linear regression equations using SPSS 20 (IBM).

3. Results
3.1 Characteristic and Composition of A. dorsata Binghami Venom
The characteristics of the honey bee venom that were freshly taken from the worker bees are white, clear, odorless, tasteless as if burning on tongue, pH (4.00 to 5.3) (table 1). About five to ten minutes at room temperature, it will turn into a powder as flour is yellowish white. Needle injection of bee venom is present on the part of the abdomen called the sting. Sting is present on the end of the abdomen. Sting of A. dorsata Binghami is blackish at the base, with a venom sacs containing fresh venom (Figure 2). Isolation of venom from 100 individial of bees produce dry bee venom 0,038 grams on average.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Fresh Bee venom</th>
<th>dry bee venom</th>
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<tbody>
<tr>
<td>pH</td>
<td>4,00 – 5,3</td>
<td>4,6 – 5,8</td>
</tr>
<tr>
<td>Form</td>
<td>Semisolid fluids</td>
<td>powder/crystals</td>
</tr>
<tr>
<td>Colours</td>
<td>Clear white</td>
<td>White yellowish</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water, ethanol and ammonium sulfate</td>
<td>Soluble in water, ethanol and ammonium sulfate</td>
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<tr>
<td>taste on the tongue</td>
<td>Burning</td>
<td>Burning with the intensity of the smaller</td>
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<td>The Phytochemicals compounds</td>
<td>- Polifenol</td>
<td>+</td>
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<td>- Flavonoid</td>
<td>+</td>
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| Table 1: Characteristics of fresh and dry bee venom of A. dorsata Binghami |
3.2 The analysis of bee venom component using SDS-PAGE

Polyacrylamide gel concentrations used in this study was 17.5%. The results of the analysis of SDS-PAGE of bee venom obtained 5 a clear tape. Molecular weight in five consecutive AD1 Ribbon was 33.53 kDa, 21 kDa, 10 kDa, 6.1 kDa and 2.67 kDa, while for AD2, it was found that ordered consecutive was 33.52 kDa, 31.21 kDa, 21 kDa, 10 kDa, 6.1 kDa and 2.67 kDa (Figure 3 and table 2).

Table 2: Molecular weight (kDal) bee venom after analyzed based on SDS-PAGE Cromatogram

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Band 1</th>
<th>Band 2</th>
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<th>Band 4</th>
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<td>33.53</td>
<td>31.21</td>
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<td>15</td>
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<td>6,1</td>
<td>4.6</td>
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3.3 The activity of the free radical DPPH Scavenging

The activity of the free radical DPPH scavenging by bee venom was isolated from fresh *A. dorsata* Binghami on natural nesting in Kombi forests (AD2) was compared with the isolation of Kaweruan Forests (AD1). Inhibitory concentration 50 (IC50) AD1 is 103,28 ppm (y = 16, 063ln (x)-24,492; R² = 0,9344) while in AD2 IC50 is 139,13 ppm (y = 16, 241ln (x)-30,151, R² = 0,8515) (Figure 2). Compared to positive control i.e. synthetic antioxidants Butyl hydroxy toluene (BHT) antioxidant activity, crude venom *A. dorsata* Binghami is still stronger based on the IC50 (Figure 4).

3.4 Cytotoxic Activity

Honey bee venom that is used to test the toxicity of the venom *A. dorsata* is crude bee venom. Cytotoxic test results showed that *A. dorsata* venom had a stronger cytotoxic activity in cell murine leukemia p388. Inhibitroy concentration 50 (IC50) of AD1 and AD2 are 36,12 µg/l, and 48,59 µg/ml, respectively. Compared with the positive control, AD2 showed stronger cytotoxic effect (Figure 5).

4. Discussion

Characteristic and composition of *A. dorsata* Binghami venom

Bee venom has been used in wound healing for centuries. From the results of the analysis of fresh venom of *A. Dorsata*, it was found that it contains polyphenols and flavonoids compounds. SDS-PAGE analysis based on molecular weight peptides revealed that fresh bee venom of *A. dorsata* Binghami contains hyaluronidase, phospholiupase, phospholipase A, inhibitor protease and mellitin. There are some bands with unknown types of peptides. The difference in molecular weight peptides were detected on the SDS-PAGE of the AD1 and AD2 indicates that the content of
bioactive materials on honey bees can be different depending on the variety and availability of plant as their food sources. Mellitin and phospholipase A was a constituent component of bee venom honey with a high toxicity compared to other components. Mellitin compiled a 30% up to 50% dry weight venom of A. mellifera while phospholipase A was approximately 10% to 12%. Our results of the SDS-PAGE with fresh bee venom A. dorsata indicate that the thickness of the band is high, not only on melitin and protease inhibitors but also on phospholipase and hyaluronidase. The thickness of the tape shows the number concentration of compounds that contained. Venom from other Apis species is similar, but even the venoms from the various races within each species are slightly different from each other. The toxicity of Apis cerana venom has been reported to be twice as high as that of A. Mellifera (Ali, 2012). With the existence of a band that has not been known, it is imperative to conduct further analysis with different methods to find out the bioactive substances.

4.1 Antioxidant Activity
Antioxidant activity of honey bee venom was analyzed through a process of reaction with using DPPH. Due to these reactions, bee venom stabilizing free radicals and reduces absorption as a consequence and DPPH radical DPPH decrease to DPPH-H. The degree of discolorisation shows the potential silencing of free radical antioxidant substance or extract with hydrogen gives. The DPPH antioxidant reacts will show a change in color from orange to yellow, with the colour intensity depending on the ability of antioxidants (Benabadji et al. 2004). In this research, it was shown radical curbs AD1 DPPH with IC50 values of 103,28 µg/ml whereas AD2 IC50 of 139,13 µg/ml. Previous research with tiobarbituric acid method (TBA) showed that at concentrations of 500 ppm poison of Apis nigrocinata is able to inhibit the oxidation of linoleic acid of 75,10%, whereas A. dorsata in a concentration of 200 ppm has been able to inhibit the oxidation of linoleic acid 80,78% (Mokosuli et al. 2013 [28, 44]).

Based on the analysis of the composition of the venom using SDS-PAGE, the content of peptide found in the venom of bees is melitin, phospholipase, phospholipase A or lyso phospholipase or antigen 5, protease inhibitors and hyaluronidase. Bee venom was also shown to significantly decrease the level of Radical Oxygen Species (ROS)-induced oxidative damage to synovial fluid proteins in a rat model of rheumatoid arthritis (Frances et al., 2014). DPPH free radical scavenging activity of the Bee Venom group was 2.8 times stronger than that of the Sweet Bee Venom group (Chull et al. 2006) [6]. The honey bee venom also possesses a considerable hydroxyl radical scavenging activity which was evaluated by its competition with dimethyl sulphoxide for OH-. Honeybee venom is found to significantly inhibit lipid peroxidation of non-enzymatic reactions. It also possesses a considerable hydroxyl radical scavenging activity, evaluated by its competition with dimethyl sulfoxide for HO (Rekka et al., 1990) [34].

Analysis of the content of the phytochemicals are known to bee venom A. dorsata Bingham can be cytotoxic on cancerous cells of murine leukemia cell line P388. Future research needs to be done for in vivo test of A. dorsata Bingham venom to develop dose and formulation curve of effectiveness against cancer cells.

4.2 Cytotoxic Activity
As with antioxidant activity, cytotoxic activity of AD1 (IC50: 36.12 µg/l) is more powerful than the AD2 (IC50: 48.59 µg/ml). Melitin are cytotoxic on cancerous cells with cell membrane damage. Bee venom may inhibit tumor cell growth (Orsolic et al., 2009) [33]. Mellitin and phospholipase A can increase the synthesis of tumor cell necrosis factors, such as cytokines and interleukin-1, stimulates the release of arachidonat acid which is produced in the process of immune response. The bee venom was able to induce apoptosis in many types of cancer cell culture. It also induces morphological changes and a decrease in the percentage of viable cells on cell culture of cervical cancer. Analysis of flow-cytometry shows that bee venom can induce the production of ROS, improving the content of cytoplasmic Ca²⁺, reduces mitochondrial membrane potential, that would eventually causes the release of cytochrome promoting activation and caspase-3 and trigger apoptosis. Bee venom also induces an increase in Fas, p53, p21 and Bax but lowers BCL-2 (Wan et al., 2008) [38]. Bee venom significantly inhibits the growth of cancer cells in the lungs. Bee venom also inhibits vascular endothelial growth factor (VEGF) that induce proliferation. (Hulha et al., 2010) [10]. Recent studies reported that bee venom possesses antimutagenic (Varanda et al., 1999) [45], proinflammatory (Surendra et al., 2011 [46], antiinflammatory (Nam et al., 2005) [47], and antinociceptive effect (Kim et al., 2013) [48]. Bee venom has also anti-cancer activity and has the capacity to kill cancer cells. The promise of this remedy exists with living honeybees, which make tumors disappear by killing cancer cells (Liu et al., 2002) [23]. The cytotoxic effect through the activation of PLA₂ by melitin is believed to be an important mechanism of anti-cancer activity of BV. Thus the content of bee venom A. dorsata Bingham can be cytotoxic on cancerous cells of murine leukemia cell line P388. Future research needs to be done for in vivo test of A. dorsata Bingham venom to develop dose and formulation curve of effectiveness against cancer cells.
Fig. Kromatogram results of the SDS PAGE. Found 5 ribbons of proteins with molecular weights in a row: 40 kDa, 25 kDa, 15 kDa, 10 kDa and 4.6 kDa. Apitoksin standard (S), marker proteins (M), the toxin a. dorsata (BV1), toxins of a. nigrocincta (BV).

**Table.** Molecular weight (kDal) bee venom after analyzed based on SDS-PAGE Cromatogram

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**4.3 Antioxidants test**

![Graph](Image)

6. Acknowledgment

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7. References


