



## ***In-vitro* antileptospiral activity of *Canarium odontophyllum* Miq. (Dabai) leaves extract**

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### **ABSTRACT**

**Aims:** To evaluate the anti-leptospiral activity of *Canarium odontophyllum* leaves against *Leptospira interrogans* serovar Bataviae and *Leptospira borgpetersenii* serovar Javanica.

**Methodology and results:** The extracts (hexane, acetone, methanol and aqueous) used in this study were tested at concentration ranging from 0.049 mg/mL to 25 mg/mL using broth microdilution method. Percentage inhibition (%) was obtained through OD reading at 400 nm. Only methanol extract was incubated with *Leptospira* to observe population changes under dark field microscope prior to subjected for DNA damaging studies through gel electrophoresis of genomic DNA. Methanol extract showed the highest percentage inhibition of 66% against *L. interrogans* serovar Bataviae and 74% against *L. borgpetersenii* serovar Javanica. The IC<sub>50</sub> value of methanol extract was 4.60 mg/mL and 2.25 mg/mL against serovar Bataviae and serovar Javanica, respectively. Both *Leptospira* culture which was treated with IC<sub>50</sub> value of methanol extract showed drastic decrease in population compared to untreated *Leptospira* for both serovar. There was no DNA damage towards serovar Bataviae. However, serovar Javanica exhibited DNA damage as observed from the presence of DNA fragmentation on the gel electrophoresis.

**Conclusion, significance and impact of study:** These findings confirmed that methanol leaves extract from of *Canarium odontophyllum* has a potential to control leptospirosis.

**Keywords:** anti-leptospiral, *Leptospira interrogans*, *Leptospira borgpetersenii*, *Canarium odontophyllum*

### **INTRODUCTION**

Leptospirosis is a re-emerging zoonotic disease where the first case recorded in Malaysia was in 1925. This disease occurs due to the bacterial infection by pathogenic *Leptospira* species which is carried by animals and transmitted to humans through various mechanisms (Lim *et al.*, 2011).

Genus *Leptospira* is divided into two species which is pathogenic and non-pathogenic or saprophytic. Pathogenic *Leptospira* such as *Leptospira interrogans* is the cause of Leptospirosis disease while saprophytic *Leptospira* such as *L. biflexa* are the living organisms that are free in water and soil and not infecting animal and human. *Leptospira* sp. is a spiral Gram-negative bacterium and has internal flagella (Johnson, 1996). *Lepstospira* sp. has a diameter of 0.1 µm and a length about 6 to 20 µm. The cells have pointed ends which either one or both of the ends will bend into a distinctive hook. Movement of *Leptospira* sp. produced by two

flagella that rotate over the membranes and these bacteria are mostly carried by small rodents and mammals which infect humans through urine containing *Leptospira* bacteria either directly or indirectly (Levett, 2001).

According to the latest statistics released by the Ministry of Health Malaysia, there were 31,771 cases of leptospirosis reported in 2011 to 2016 which lead to 396 deaths. According to the Ministry of Health Malaysia through MyHealth portal, the signs and symptoms of this illness begin with fever, chill, body ache and headache in about 75% to 100% of the patient. 25% to 35% of them are coughing and about 7% to 40% of the patients may have muscle aches, spleen or liver enlargement, swelling of the gland, sore throat, muscle spasm and rashes.

*Canarium odontophyllum* Miq. or known as dabai by local people in Sarawak who come from the *Burseraceae* family are trees that can be found in tropical rainforests in Sarawak, Malaysia (Ding and Diana, 2013). The dabai tree grows vertically to a height of 30 to 50 meters and

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has a large pinnate leaf size. Phytochemical studies on dabai leaves showed that it contains flavonoids, tannins, saponins, terpenoid and phenolic compounds in aqueous, acetone and methanol extracts (Basri and Nor, 2014). Acetone extract of *Canarium odontophyllum* has been proven to act as an anti-bacterial agent whereas methanol extract of this leaf also has a potential anti-MRSA agent (Basri and Nor, 2014; Basri *et al.*, 2014c). The aqueous extract has been shown to reduce sugar level in diabetic rat (Saari *et al.*, 2017). Apart from its leaf, study on stem bark and fruit of *Canarium odontophyllum* tree has also been conducted and have been proven as anti-oxidant (Shakirin *et al.*, 2010), anti-cancer agent (Basri *et al.*, 2014a), anti-atherosclerotic (Shakirin *et al.*, 2012), anti-microbial and anti-yeast (Basri *et al.*, 2014b). However, studies on *Canarium odontophyllum* leaf against *Leptospira* bacteria have not been conducted yet. Thus, with various pharmacological activities that have been reported on leaf extract from *C. odontophyllum*, its potential against *Leptospira* sp. is necessary to be carried out.

## MATERIALS AND METHODS

### Chemicals

*Leptospira* sp. Ellinghausen-McCullough-Johnson-Harris (EMJH) enrichment media Difco™ (Becton, Dickinson and Co.USA), *Leptospira* EMJH media Difco™ (Becton, Dickinson and Co. USA), Tris Borat EDTA (Next Gene Scientific Sdn Bhd Malaysia), Agarose (Next Gene Scientific Sdn Bhd Malaysia), DNA dye GelRed™ (Biotium, California), Gel Loading Dye Blue (Thermo Scientific, USA), 100 bp Ladder DNA (Thermo Scientific, USA), Primer LipL32 (N-gene IDT, Berlin), Econo Taq® Green Master Mix PCR (Lucigen, Middleton).

### *Leptospira* culture sample

*Leptospira* culture used in this study were the reference strain which was *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica, pathogenic *Leptospira* supplied by Unit of Biotechnology, Institute of Medical Research, Kuala Lumpur.

### DNA extraction

The DNA was extracted following Qiagen DNA Extraction Kit (USA) protocol. Extracted genomic DNA was used as template for PCR with modification on the primers to detect LipL32 gene generating the 786 base pair product. The PCR was performed with 2.5 units of the Taq DNA polymerase (Fermentas AB, Lithuania) in a reaction mixture (100 µL) containing dNTPs (200 µM) and 2.5 mM MgCl<sub>2</sub>, subjected to 40 cycles of 50 sec at 94 °C (denaturation), 1 min at 62 °C (annealing) and 1 min at 72 °C (extension). The amplified PCR product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany). About 2.3 g of EMJH *Leptospira* spp. Difco™ basic media was dissolved with 900 mL

distilled water in a 1 L Scotch bottle at pH 7.5, autoclaved for 15 min, left at room temperature overnight and filtered through a 0.2 µm filter unit. About 100 mL EMJH *Leptospira* spp. Difco™ enriched media was added to the 900 mL basic media prepared earlier. Next 5 mL of the media was aliquoted into each of the 10 mL culture tubes and 5-fluorouracil (200 µg/mL) was also added to it. These culture tubes were then kept in 4 °C until used.

### Polymerase chain reaction (PCR)

The PCR was conducted to make sure the *Leptospira* used was a pathogenic *Leptospira* by using primer LipL32. This assay was done using 50 µL reaction mixture that consisted of 25 µL Econo Taq® Green Master Mix, 20 µL of sterile distilled water 1 µL of each and forward primer and 3 µL of template DNA of each serovar. Base pair of LipL32 forward was 5'- GTC GAC ATG AAA CTT TCG ATT TTG-3' and base pair of LipL32 reverse primer was 5'- CTG CAG TTA CTT AGT CGC GTC AGA AGC-3'. The mixture was mixed using micro centrifuge and performed in the Thermocycler with the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 sec, extension at 72 °C for 30 sec and a final extension at 72 °C for 6 min. The PCR product were checked for amplification by electrophoresis using 1.5% agarose gel and visualized by gel documentation system and photographed. (Cheema *et al.*, 2007)

### Percentage inhibition (%) test

Percentage inhibition test was conducted to test various concentrations ranging from 0.049 mg/mL to 25 mg/mL of *Canarium odontophyllum* leaves extracts in inhibiting the activity of *Leptospira* sp. using broth microdilution technique with 96-well plate (Murray and Hostenhal, 2004). One hundred microliter *Leptospira* in EMJH media was poured in all well (one plate for one serovar). Each row is designated for various concentrations with same solvent of the extract (methanol, acetone, aqueous, and hexane). In each row, 50 µL of various concentrations of leaf extract at 0.049 mg/mL, 0.098 mg/mL, 0.195 mg/mL, 0.391 mg/mL, 0.781 mg/mL, 1.563 mg/mL, 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL and 25 mg/mL was added horizontally and incubated for three days at 30 °C in the dark condition. After incubation time, the plate was read by spectrophotometer at the wavelength of 400 nm (Stoddard *et al.*, 2014) in triplicate and the average OD of test was calculated. The percentage of inhibition of *Leptospira* growth by *Canarium odontophyllum* extract was calculated through the following formula (Wang *et al.*, 2010):

$$\text{Inhibition Percentage (\%)} = \frac{\text{OD (control)} - \text{OD (test)}}{\text{OD (control)}} \times 100$$

Where,

OD (control) = OD400 reading average for control sample  
OD (test) = Average OD400 reading for test sample

Note: Percentage of inhibition is 0 or no inhibition if the result is negative

### Dark Field Microscopy

Before *Leptospira* treated with IC<sub>50</sub> of the most effective extract, the culture was observed under dark field microscope (Murray and Hospenthal, 2004). Then, the culture was incubated with IC<sub>50</sub> of the most effective extract for each serovar and incubated for three days at 30 °C in the dark condition. After the incubation time, drop of sample was placed in the slide and observed under dark field microscope again to check the population changes by comparing with the untreated culture. This procedure was done to compare whether there were any changes before and after the treatment of the extract with *Leptospira* culture.

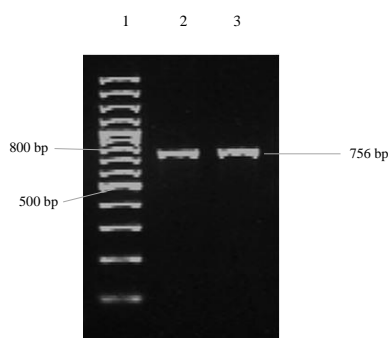
### Genomic DNA damage

IC<sub>50</sub> of the most effective *Canarium odontophyllum* leaves extract was added in the *Leptospira* culture in EMJH media and incubated for three days at 30 °C in the dark condition. After incubation time, genomic DNA was isolated according to manufacturer instruction (HiYield™ Genomic DNA (Bacteria) Mini Kit). For control, genomic DNA was isolated from *Leptospira* culture without extract. Genomic DNA sample (2 µL) mixed with 4 µL of dye was loaded on a 1% agarose gel with 3 µL ethidium bromide. The sample was run for 1 h at 50 mV. Gel was observed under UV light and photographs were taken using gel documentation system. This test was done to observe whether the extract could cause fragmentation and degradation of *Leptospira* genomic DNA (Ahsan *et al.*, 2012).

## RESULTS

### Polymerase chain reaction (PCR)

Both *Leptospira* used in this study which were *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica were confirmed pathogenic with the presence of band at 756bp obtained with LipL32 based primers as shown in Figure 1.



**Figure 1:** PCR amplification of LipL32 gene.

Lane 1: 100bp DNA ladder  
 Lane 2: PCR product of *L. interrogans* serovar Bataviae  
 Lane 3: PCR product *L. borgpetersenii* serovar Javanica

### Percentage (%) of *Leptospira* inhibition

The percentage inhibition of *Canarium odontophyllum* leaves extract against *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica were shown in Table 1 and Table 2 respectively. After the calculation using the OD, the result showed that methanol leaves extract from *C. odontophyllum* exhibited the highest percentage of *Leptospira* inhibition for both serovar. Methanol extract displayed a percentage of inhibition of 66% for *L. interrogans* serovar Bataviae and 74% for *L. borgpetersenii* serovar Javanica at the concentration of 25 mg/mL. However, all extracts namely acetone, aqueous and hexane also showed inhibitory effect against *Leptospira* but not as effective as methanol extract. Hexane extract exhibited the lowest percentage of *Leptospira* inhibition which was only 25% for *L. interrogans* serovar Bataviae and 30% for *L. borgpetersenii* serovar Javanica at 25 mg/mL. Thus, methanol extract was chosen for the further investigation.

**Table 1:** Percentage inhibition of *L. interrogans* serovar Bataviae.

Extract concentration of <i>Canarium odontophyllum</i> (mg/mL)	Percentage (%) of <i>Leptospira</i> inhibition in various concentration and solvent			
	Methanol	Acetone	Aqueous	Hexane
0.049	0	0	0	0
0.098	4	1	0	0
0.195	5	3	0	0
0.391	12	8	6	0
0.781	26	15	10	4
1.563	33	21	18	6
3.125	47	24	21	11
6.25	51	33	27	16
12.5	63	41	38	22
25	66	46	40	25

**Table 2:** Percentage inhibition of *L. borgpetersenii* serovar Javanica.

Extract concentration of <i>Canarium odontophyllum</i> (mg/mL)	Percentage (%) of <i>Leptospira</i> inhibition in various concentration and solvent			
	Methanol	Acetone	Aqueous	Hexane
0.049	0	0	0	0
0.098	7	2	2	0
0.195	18	7	11	0
0.391	27	16	20	4
0.781	38	23	25	10
1.563	41	28	32	14
3.125	58	44	38	20
6.25	63	53	44	24
12.5	71	55	50	28
25	74	58	53	30

### IC<sub>50</sub> value of methanol extract for each serovar

From the result above, the graph was plotted and the concentration of the most effective extract which was

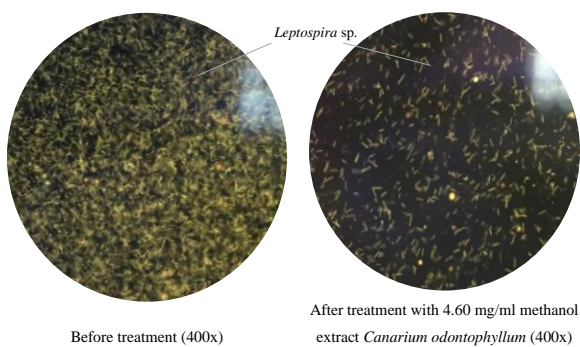
methanol extract that inhibits 50% of the population for each *Leptospira* serovar was identified. Thus, table below showed the IC<sub>50</sub> value of methanol extract for each serovar.

**Table 3:** IC<sub>50</sub> value of *Canarium odontophyllum* methanol leaves extract.

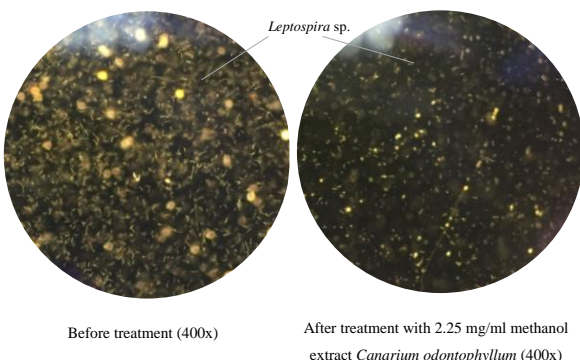
<i>Leptospira</i> sp.	IC <sub>50</sub> value (mg/mL)
<i>L. interrogans</i> serovar Bataviae	4.60
<i>L. borgpetersenii</i> serovar Javanica	2.25

***Leptospira* population changes under dark field microscope**

Reduction of *Leptospira* population in the culture after incubation time with the IC<sub>50</sub> value of methanol extract for each serovar was proven through the observation under dark field microscope as shown in Figure 2; *L. interrogans* serovar Bataviae and Figure 3; *L. borgpetersenii* serovar Javanica. From the observation, the untreated culture has more cells compared to the treated culture which indicated that the extract was able to lower the number of *Leptospira* cells.



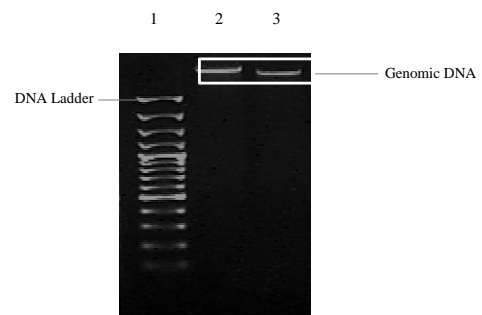
**Figure 2:** *L. interrogans* serovar Bataviae population before and after treated with IC<sub>50</sub> value of methanol extract.



**Figure 3:** *L. borgpetersenii* serovar Javanica population before and after treated with IC<sub>50</sub> value of methanol extract.

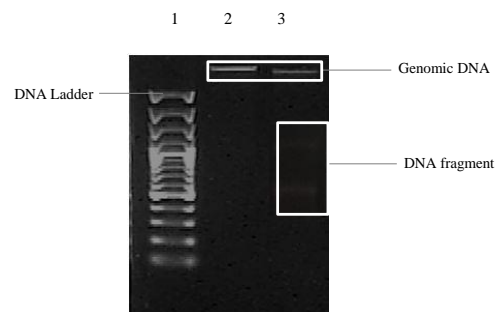
**Genomic DNA damage**

Genomic DNA degradation was observed in *L. borgpetersenii* serovar Javanica (Figure 5) but not in *L. interrogans* serovar Bataviae (Figure 4) by IC<sub>50</sub> value of *Canarium odontophyllum* methanol leaves extract because the DNA fragmentation only happen on the gel which contain genomic DNA of *L. borgpetersenii* serovar Javanica. Thus, proves that this IC<sub>50</sub> value of extract could only react and damage the DNA of *L. borgpetersenii* serovar Javanica but not *L. interrogans* serovar Bataviae.



**Figure 4:** Genomic DNA *L. interrogans* serovar Bataviae gel electrophoresis.

Lane 1: Ladder DNA  
 Lane 2: Genomic DNA *L. interrogans* serovar Bataviae without IC<sub>50</sub>  
 Lane 3: Genomic DNA *L. interrogans* serovar Bataviae after incubated with 4.60 mg/mL



**Figure 5:** Genomic DNA *L. borgpetersenii* serovar Javanica gel electrophoresis.

Lane 1: Ladder DNA  
 Lane 2: Genomic DNA *L. borgpetersenii* serovar Javanica without IC<sub>50</sub>  
 Lane 3: Genomic DNA *L. borgpetersenii* serovar Javanica after incubated with 2.25 mg/mL

**DISCUSSION**

*Leptospira* sp. is the cause of Leptospirosis. *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica

were chosen in this study because both these serovar were the most dominant serovar present in rat in Kuala Lumpur (Benacer *et al.*, 2013) and Peninsular Malaysia (Benacer *et al.*, 2016) Thus, these serovar were chosen to be significant to show infection in Malaysia.

As we know, *Leptospira* is divided into two which is pathogenic and non-pathogenic (saprophytic) so to differentiate this two, we have to do polymerase chain reaction (PCR). The primer that was used to detect pathogenic *Leptospira* is LipL32 because LipL32 is one of outer membrane protein that is only present in pathogenic *Leptospira* whether *in-vivo* or *in-vitro* (Haake and Levett, 2015) Based on PCR product results, the presence of band at 756 bp when using LipL32 (Cheema *et al.*, 2007) proved that both *Leptospira* cultures were found to be pathogenic.

*Canarium odontophyllum* or known as dabai are very popular among the people in Sarawak and the fruit is often used as food. However, its leaves have not been used until the study of dabai leave extract turned out to be an anti-bacterial agent especially towards *Staphylococcus aureus* (Basri and Nor, 2014), an anti-MRSA agent (Basri *et al.*, 2014c), blood glucose reducing agent (Saari *et al.*, 2017), vasorelaxant effect (Basri *et al.*, 2018) and anti-cancer activity to colorectal cancer (Basri *et al.*, 2015). Scientists were interested to use the leaves because they can be easily obtained from its large tree which produce many leaves regardless of season unlike its seasonal-grown fruit (MARDI, 2014).

From percentage inhibition result, different solvent of extract gives a different percentage of inhibition which is methanol extract gives a highest percentage of inhibition while hexane gives a low percentage of inhibition. This is because different solvents have the different ability to extract active ingredients from plants. The use of methanol as a solvent can dissolve various active compounds either polar or non-polar (Basri *et al.*, 2014b) This was proved by the presence of many phytochemical constituent study of *C. odontophyllum* methanol leaves extract by Basri and Nor (2014) such as flavonoids, saponins, tannins, terpenoid and phenolic compounds. However, phytochemical studies for hexane extracts of *C. odontophyllum* leaves have not been carried out yet. According to previous studies using crude extracts of *C. odontophyllum* leaves, methanol extract was also the most effective extract as an antibacterial agent (Basri and Nor, 2014) but this study is not conducted against *Leptospira* sp.

IC<sub>50</sub> value was different for each serovar because every *Leptospira* serovar has different lipopolysaccharide expression on its surface (Adler *et al.*, 2010) Furthermore, *L. interrogans* is a more virulent species of *L. borgpetersenii* (Lehmann *et al.*, 2014) so it is possible that it requires higher extract concentration to inhibit 50% of its population. Bulach *et al.* (2006) in his study stated that external surface proteins that contributed to *Leptospira's* efficacy such as LigA, LigB and LigC were present in *L. interrogans* but for *L. borgpetersenii* only had LigB which made them less virulent but still able to infect and become pathogenic to humans.

Observation under a dark field microscope is very important to support the percentage of inhibition result and IC<sub>50</sub> and also one of the most important methods to detect the presence of *Leptospira* in the culture. The population of treated *Leptospira* cultures had less cell compared untreated culture. Even though dark field microscope results are only able to see population changes in terms of qualitative but according to study done by Terpstra (2003) emphasized that although observation of *Leptospira* under dark-field microscopy are conventional techniques with qualitative calculation, it is the most useful technique for showing and determining the population of *Leptospira*. This technique can be improved by using a counting chamber but the motile nature of *Leptospira* can make the calculation inaccurate.

There are many plant extracts that have shown to cause cell death involving bacterial DNA fragmentation. For example, the ethanol extract of *Syzygium cumini* leaves has caused fragmentation of genomic DNA of *Vibrio cholerae* (Ahsan *et al.*, 2012). Methanol and aqueous extracts from *Eclipta alba* and *Phyllanthus amarus* also resulted in fragmentation of the genome DNA of *Leptospira* sp. (Chandan *et al.*, 2012). This is probably because the extracts activated the intracellular signals and trigger the endonuclease to carry out bacterial DNA damage. However, further investigation is needed to explain the exact mechanism of this extract as an anti-leptospirosis agent.

## CONCLUSION

The methanol extract from *Canarium odontophyllum* leaves exhibited an anti-leptospirosis effect especially towards *L. borgpetersenii* serovar Javanica and could be used to control infection caused by leptospirosis.

## ACKNOWLEDGEMENTS

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