EVALUATION OF ANTIFUNGAL ACTIVITY OF *NEOLAMARCKIA MACROPHYLLA* LEAF EXTRACT

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EVALUATION OF ANTIFUNGAL ACTIVITY OF NEOLAMARCKIA MACROPHYLLA LEAF EXTRACT

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A report submitted in partial fulfillment of the Final Year Project 2 (STF 3015) course

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2016
Acknowledgement

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Declaration

I, Michelle Ngassy Mering, hereby declare that all the writing in this dissertation is my original work except for some quotes which I have stated its source of origin.

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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>&amp;</td>
<td>Percent</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
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<td>mg/mL</td>
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Evaluation of Antifungal Activity of *Neolamarckia macrophylla* leaf extract

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**ABSTRACT**

*Neolamarckia macrophylla* (Roxb.) Bosser is from genus *Neolamarckia* of the family *Rubiaceae*. This plant is known as Red Kelampayan among the locals. It is an important tropical tree chosen for reforestation in Malaysia. The aims of this research were to extract the antifungal properties of *N. macrophylla* leaf using aqueous and methanol extraction followed by the evaluation of antifungal activity of the leaf extracts against *Aspergillus flavus*, *Aspergillus niger* and *Fusarium solani*. Extracts of different concentration (12.5 mg/mL, 25 mg/mL, 50 mg/mL and 100 mg/mL) were spread uniformly on the potato dextrose agar to evaluate the fungi growth using the poisoned food technique. The results were compared with the normal growth of the fungi in potato dextrose agar. All of the results showed that there is inhibitory effect on the fungi exhibiting percentage within ranges of 4-80%. The most effective extraction method to check antifungal activity of *N. macrophylla* was the aqueous extraction of which the highest percentage inhibition obtained was 80%. Further studies are needed so that the inconsistency of the results obtained may be reduced and also to improve on the evaluation of the potential of *N. macrophylla* as antifungal agents.

**Keywords:** antifungal activity, leaf extracts, *Neolamarckia macrophylla*
Evaluation of Antifungal Activity of Neolamarckia macrophylla leaf extract

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ABSTRAK

Neolamarckia macrophylla (Roxb.) Bosser adalah dari genus Neolamarckia famili Rubiaceae. Dalam kalangan penduduk setempat, tumbuhan ini dikenali sebagai kelampayan merah. Kayu tropika ini penting sebagai sumber kayu untuk penghutanan semula. Objektif utama kajian ini dijalankan adalah untuk memperoleh metabolit sekunder daripada daun N. macrophylla menggunakan ekstrasi akua dan metanol. Selain itu, objektif kajian ini juga adalah untuk menilai aktiviti antikulat metabolit sekunder daun N. macrophylla terhadap Aspergillus flavus, Aspergillus niger dan Fusarium solani. Ekstrak-eks trak berlainan kepekatan (12.5 mg/mL, 25 mg/mL, 50 mg/mL dan 100 mg/mL) diratakan sama rata atas agar-agar kentang dekstrosa untuk menilai aktiviti antikulat dengan menggunakan 'poisoned food technique'. Aktiviti antikulat metabolit sekunder daun ini dibandingkan dengan ketumbuhan biasa kulat di atas agar-agar kentang dekstrosa. Kesemua hasil kajian menunjukkan terdapat rencatan pada ketumbuhan kesemua kulat dalam lingkungan 4-80%. Kaedah ekstrasi yang paling berkesan untuk memperoleh metabolit sekunder daripada daun N. macrophylla adalah ekstrasi akua di mana peratusan rencatan yang paling tinggi ialah 80%. Analisis lanjutan perlu dijalankan untuk mengurangkan kadar tidak konsisten hasil yang diperoleh dan juga untuk mengenalpasti dengan lebih lanjut kebolehan N. macrophylla sebagai ejen antikulat metabolit sekunder.

Kata kunci: aktiviti antikulat, ekstrasi daun, Neolamarckia macrophylla
1.0 Introduction

The tropical rain forests of Malaysia are biologically diverse. The trees or vegetation especially, are very important. Not only the trees give habitats for other forest species (Canon et al., 1998) but trees also provide resources for timber products and non-timber products.

*Neolamarckia macrophylla*, a member of the family Rubiaceae is one of the important tropical trees chosen for reforestation in Malaysia (Chang et al., 2014). The tree originated from tropical rain forest such as Malaysia, Indonesia, Vietnam and the Philippines. Besides, this species of tree is also found in Thailand and China before being introduced to South Africa, Puerto Rico and Taiwan (Halawane et al., 2011). The preferential for this tree is high because of their adaptability and economic profitability (Irawan & Purwanto, 2014).

It is a strong and fast growing tree that is able to adapt on degraded land, resistant to serious diseases and pests and has many potential in the production of plywood, canoe and timber trade (Chang et al., 2014). It has hairy and reddish green in colour leaves (Tan et al., 2014) and they have been used as mouth gargles (Halawane et al., 2011). Even so, the research done on the antifungal activity of leaf extract of this species is very limited. According to Patel et al. (2011), *Neolamarckia cadamba* leaf extract would be helpful in treating various kinds of diseases such as cough, wounds and uterine complaints. Although *N. cadamba* and *N. macrophylla* are classified under the same genus, the potential of *N. macrophylla* as a source of bioactive compound that possess antifungal activity have yet to be studied.

In addition, as a timber tree, limited parts of the tree are used and other parts of the plant will be a wastage. Leaves are not sawn products but because the concentration of
antifungal compounds may vary among the different parts of the plant (Shetty et al., 1987), the leaves of *N. macrophylla* may have the medicinal values. Moreover, traditional medicine is said to be cheaper and much more effective compared to synthetic drugs (Mathur et al., 2011). By doing the research, the antifungal activity of *N. macrophylla* leaf parts is able to be evaluated and used to assess the potential of new alternative for drug development. If the *N. macrophylla* proves to be a medicinal plant, new compounds found will help to reduce the production of synthetic antimicrobial medications. More research are able to be done in Malaysia, making an easy access of information on the plantation planted in own country.

Hence, the objectives of this research:

1. To extract the antifungal properties of *Neolamarckia macrophylla* leaf using aqueous and methanol extraction
2. To evaluate the antifungal activity of the leaf extracts against *Aspergillus flavus, Aspergillus niger,* and *Fusarium solani*
2.0 Literature Review

2.1 Taxonomy of Neolamarckia macrophylla

Neolamarckia macrophylla (Roxb.) Bosser is from the genus Neolamarckia of the family Rubiaceae (The Plant List, 2010). This plant is locally known as Red Kelampayan in Malaysia. Table 2.1 shows the detailed classification of N. macrophylla.

<table>
<thead>
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<th>Kingdom</th>
<th>Plantae</th>
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<tr>
<td>Subkingdom</td>
<td>Tracheobionta - vascular plants</td>
</tr>
<tr>
<td>Superdivision</td>
<td>Spermatophyta - seed plants</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta - flowering plants</td>
</tr>
<tr>
<td>Subclass</td>
<td>Asteridae</td>
</tr>
<tr>
<td>Order</td>
<td>Rubiales</td>
</tr>
<tr>
<td>Family</td>
<td>Rubiaceae - madder family</td>
</tr>
<tr>
<td>Genus</td>
<td>Neolamarckia</td>
</tr>
<tr>
<td>Species</td>
<td>Neolamarckia macrophylla (Roxb.) Bosser</td>
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<tr>
<td>Synonyms</td>
<td>Nauclea macrophylla Roxb., Bancalus macrophyllus (Roxb.) O. Kuntze, Anthocephalus macrophyllum (Roxb.) Havil</td>
</tr>
</tbody>
</table>

This tree is a type of fast growing tree that has the potential of becoming the future wood supply because of its straight trunk with long clear lobe (Cahyono et al., 2015). It grows on locations with height of 10-1000 meter above sea level and its height is able to reach 40 meter with a stem circumference of more than 150 centimeter (diameter greater than 50 centimeter).
The tree has distinctive characteristics because of its wood. Its wood is red, has smooth wood texture and straight grain. Besides, the wood is considered strong and used as ‘plywood, furniture, canoe and light construction’ (Tan et al., 2014). In addition, the tree bore reddish brown in colour fruits when they are ripe.

The leaves are hairy and the basal leaves are sharp-pointed. Both the young leaf buds and the primary leaf veins of this species are red in colour (Halawane et al., 2011).

According to Halawane et al. (2011), in Indonesia, the barks of the trees are used as stamina enhancer drug, to reduce the cholesterol level and also tiredness. Even so, little information is known about the leaf, especially their antifungal agents because there is lack of scientific research on this part of the tree.

2.2 Medically Important Fungi

Fungi are capable of colonizing and infecting humans and other organisms, causing diseases. Aspergillus flavus, Aspergillus niger and Fusarium solani are examples of medically important fungi that have been widely used in the evaluation of antifungal of leaf extracts (Naz & Bano, 2007; Srichana et al., 2009; Mahmoud et al., 2011; Bhardwaj, 2012; Moorthy et al., 2013).

Aspergillus flavus produce aflatoxin which is carcinogenic and able to damage the internal organs (Srichana et al., 2009). Aspergillus niger is able to cause cutaneous infections (Loudon et al., 1996) and also pulmonary disease (Person et al., 2010). Fusarium solani were reported to be dominant causative agent for fusarial keratitis compared to the other Fusarium species found (Doczi et al., 2004).

Due to the increasing development of drugs in treatment of infectious diseases, fungi have developed resistance (Mahlo et al., 2010) and besides, side effects of some antimicrobial agents have been identified (Phongpaichit et al., 2004). Therefore, it is
necessary that new antifungal agents are identified to developed new and effective drugs with fewer side effects.

2.3 Antifungal Activity of Timber Plants

Search for new antifungal agents from plants are now moving towards the timber species (Mondali et al., 2009; Mahlo et al., 2010; Patel et al., 2011). A research on the antifungal activity of selected commercial Malaysia timber species done by Kawamura et al. (2010) have revealed that most of the species are effective against either Gloeophyllum trabeum (brown-rot fungi) and Pycnoporus sanguineus (white-rot fungi). However, the antifungal activity of _N. macrophylla_ is yet to be studied.

The phytochemical study of the _Neolamarckia cadamba_ showed that it constitutes secondary metabolites of indole alkaloids, sapogenins, saponins, steroids, terpenes and terpenoids (Prajapati et al., 2007). Plant secondary metabolites compounds and their derivatives are the ones that is extracted out and used to produce natural products drug because these compounds have diverse biological activities such as antimicrobial, antidiarrheal, anti-diabetic and anti-inflammatory activites (Compean & Ynalvez, 2014).

_N. cadamba_ is another plant species from the genus _Neolamarckia_ of the family Rubiaceae. Based on previous research, an evaluation of antifungal activity of _N. cadamba_ leaf extract has been done by Patel et al. (2011). Patel et al. (2011) concluded that _N. cadamba_ leaf extract would be helpful in treating various kinds of diseases. The leaf extract of _N. cadamba_ is said to be medicinally important for ulcer, wounds, aphtha, uterine complaints (Bhutya, 2011) and in the treatment of diabetes (Ahmed et al., 2011).

2.4 Evaluation of Antifungal Activity

Leaf extracts of various plant species have been widely investigated for their...
antifungal properties. Phongpaichit et al. (2004) had done an evaluation on the leaf extracts of *Cassia alata*, *Cassia fistula* and *Cassia tora* against *Microsporum gypseum*, *Trichophyton rubrum* and *Penicillium marneffei*. Jagessar et al. (2008) had investigated the antifungal activity of leaf extracts of *Momordica Charantia* against *Candida albicans*. Meanwhile, Srichana et al. (2009) had identified the effect of betel leaf extract on the growth of *Aspergillus flavus* and *Fusarium verticillioides*. According to Shai et al. (2009), leaves of some plant species have showed higher activity against pathogenic fungi compared to other parts of the plants.

There is a lot of method for the antifungal assay, for example agar disc diffusion assay and poisoned food technique (Das et al., 2010). Agar disc diffusion assay is simple and easy to perform (Macura, 1993; Barry & Brown, 1996). The disadvantages are that this method is less reproducible and less standardized (Gould, 2000). Poisoned food technique is generally used for the evaluation of antifungal activity (Grover & Moore, 1962). This method for the antifungal evaluation of plant extracts against pathogenic fungi has been used for many years (Wang et al., 2004; Mohana & Raveesha, 2007; Udin et al., 2010; Kumar & Tyagi, 2013). The poisoned food technique is one of screening method that is used to calculate the percentage of mycelial growth, which indicates the ability of the fungi to grow on the treatment media. The plant extracts are considered potent when the percent of inhibition is larger than 50%.

Two common alcoholic solvents used for extraction of active principles from the leaf are ethanol and methanol (Cowan, 1999). Ethanol is non-polar while methanol is a polar organic solvent. According to Ukaoma et al. (2013), methanol extracted more bioactive compounds compared to ethanol likely due to methanol has higher volatility compared to ethanol.

The effect of plant material differs based on its origin, extraction techniques,
3.0 Materials and Methods

The overview of the methodology for the evaluation of antifungal activity of leaf extracts of *N. macrophylla* are shown in Figure 3.1.

![Diagram](image)

*Figure 3.1. Overview of the methodology for the evaluation of antifungal activity of leaf extracts of *Neolamarckia macrophylla*.**
solvent concentration and polarity as well as the secondary metabolites of the extract (Ncube et al., 2008). Maceration technique is used in this process where the solvents diffuse into the solid plant material and solubilize compounds having the same polarity (Maiola et al., 2014).

Standard deviation are obtained to identify whether the results are consistent or vice versa. Low standard deviation values reflect consistency and high standard deviation reflect inconsistency. Based on Essghaier et al. (2012), to reduce the inconsistency, the mechanisms involved in the biocontrol activity of fungi need to be determined.
3.1 Plant Material

Fresh leaves of *N. macrophylla* were taken from the Forest Genomics and Informatics Laboratory, Department of Molecular Biology, UNIMAS.

3.2 Preparation of Extracts

The extracts were prepared based on procedures by Patel *et al.* (2011). The fresh leaves were washed two to three times on tape water and distilled water. Then, it was surface sterilized with 90% ethanol. The leaves were dried under shade and powdered.

3.2.1 Aqueous Extraction

Approximately 10 gram of pulverized plant material was extracted for 3 days by soaking it in 100 mL autoclaved water. The extracts were then filtered through filter paper, after which the filtrates were left to dry at 40-55°C. The concentrated extracts were then weighted (1.396 g). The extracts were dissolved in 2.5% dimethyl sulfoxide (DMSO) to have a concentration of 100 mg/mL. It was then stored in a Falcon tube at 4°C until required.

3.2.2 Methanol Extraction

The same method were applied for the methanol extraction. About 10 gram of pulverized plant material was extracted for 3 days by soaking it in 100 mL 70% methanol. Next, the extracts were filtered through filter paper. Then, the extracts were dried at 40-55°C by using the oven. Sticky mass of the extracts were weighted (1.747 g) and dissolve in DMSO to achieve a concentration of 100 mg/mL. It was then stored in the refrigerator until further use.
3.3 Preparation of Culture Media

The Potato dextrose agar (PDA) was prepared by dissolving 39 grams of PDA into 1000 mL distilled water. The media were sterilized by autoclaving at 15 lbs pressure at 121°C for 20 minutes.

3.4 Test Fungi

Fungi species of Aspergillus flavus and Aspergillus niger were collected from the Molecular Genetics Laboratory, Department of Molecular Biology, UNIMAS. Fusarium solani was obtained from Virology Laboratory, Department of Molecular Biology, UNIMAS.

3.5 Preparation of Fungal Inoculum

At the center of the PDA pour plates, 5-7 days old fungi were transferred and incubated at 25°C for 5-7 days. After 5-7 days, young and active growing colonies of fungi were ready to be used.

3.6 Antifungal Activity of Leaf Extracts

Required concentrations of aqueous extract were obtained by dissolving the extract in 2.5% DMSO. Aqueous extract of concentration 100 mg/mL was further diluted to the concentration of 50 mg/mL. Extract of concentration 50 mg/mL was diluted to concentration of 25 mg/mL and extract of concentration 25 mg/mL was diluted to the concentration of 12.5 mg/mL.

The antifungal activity was evaluated using the poisoned food technique (Amadioha, 2000). Approximately 100 μL of each of the extracts were pipetted and spread uniformly using a glass spreader onto the PDA plate. Mycelium block of each fungus were prepared
by punching the 5-7 days old fungal culture using equal size of pipette tips (75 mm). The mycelium block were then put at the center of the plates. In order for the mycelium to get greater contact with the culture medium, the block was put in an inverted position (Uddin et al., 2010). The inoculated plates were incubated at 25°C. The same methods were used for methanol plant extracts. The mycelium block was also grown on PDA without extracts as the negative control. The diameters of fungal colonies were measured after 5 days of incubation and there were three replicates for each of the extracts and control. The average of the replicates was taken as the colony diameter of the fungus. The percentage of mycelia growth inhibition were calculated as below (and shown as in Figure 3.2):

\[ I = \frac{(C-T)}{C} \times 100 \]

Where,

I = Percentage of mycelial growth
C = Diameter of fungal colony (mean) in control
T = Diameter of fungal colony (mean) in treatment
Figure 3.2. Diameter showing the growth of fungi

(a) $C \text{ mm}$: Diameter of fungal colony in control (b) $T = \text{ Diameter of fungal colony in treatment}$
4.0 Results and Discussion

The leaf extract obtained from *N. macrophylla* was sticky and dark brown in colour, with 10% w/w yield extract for both aqueous and methanol extract. The evaluation of the antifungal activity for both of the leaf extract were tested *in vitro* by assessing the radial growth (mm) of *Aspergillus flavus*, *Aspergillus niger* and *Fusarium solani* and their percentage inhibition. The results indicate that the growth of the fungi were inhibited by the aqueous and methanol leaf extract and its inhibition were quantitatively shown through percentage inhibition. Figure 4.1 shows the antifungal test on *A. niger* using aqueous extract with concentration 12.5 mg/mL. Both of the aqueous and methanol solvent shows that there is statistical significant (p<0.05) against all of the tested fungi.

![Figure 4.1. Antifungal test by using aqueous extraction of concentration 12.5 mg/mL. Picture taken after 3 days of incubation at room temperature. (a) Non-inverted plate (b) Inverted plate](image-url)
4.1 Comparison of the Antifungal Activity of Leaf Extracts of *N. macrophylla* Leaf Extracts Within the Same Group of Fungi

As shown in Table 4.1, normal *A. flavus* colony diameter grew unto average of 44 mm. For the aqueous extract, it shows that 50 mg/mL concentration of the *N. macrophylla* is stronger compared to the other aqueous extraction concentration. The diameter of the colony is only 19 mm with 57% inhibition. For methanol extract, lowest concentration (12.5 mg/mL) shows highest percentage inhibition with 48%. Lowest concentration to possess higher antifungal activity is possible. This is in agreement with research conducted by Naz and Bono (2012) where the antifungal potential of *Ricinus communis* leaf extracts shows that the leaf extract are effective even at low concentration against *A. flavus*.

*Table 4.1. The antifungal activity of leaf extracts of *N. macrophylla* leaf extract against *A. flavus* (5th day)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/mL)</th>
<th>Diameter (mm)±</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (DMSO)</td>
<td>-</td>
<td>44 ± 1.155</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous extraction</td>
<td>100</td>
<td>22 ± 4.163</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>19 ± 4.619</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>21 ± 1.732</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>30 ± 2.517</td>
<td>32</td>
</tr>
<tr>
<td>Methanol extraction</td>
<td>100</td>
<td>29 ± 1.732</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30 ± 9.165</td>
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<td>25</td>
<td>26 ± 7.000</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>23 ± 6.351</td>
<td>48</td>
</tr>
</tbody>
</table>

*Data are expressed as mean (n=3) ± standard deviation*