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Phytochemical Screening, Antioxidant and Cholinesterase Inhibitory Activities of *Neobalanocarpus heimii*'s Twigs Extract

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Neobalanocarpus heimii (Cengal) is a tropical timber plant that plays a prominent role in boat building, furniture making and houses. However, the bioactivities of this plant are limited and not thoroughly discussed. Thus, in this study, phytochemical screening, phenolic content, flavonoid content, antioxidant capacities, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition activities in methanol crude extract *N. heimii* were analysed. Qualitative phytochemical screening showed the presence of alkaloids, saponins, tannins, terpenoids and glycosides. The total phenolic content in *N. heimii* is higher than the flavonoid content, which supports the phytochemical screening results. *N. heimii* crude extract showed a higher free radical scavenging activity value against DPPH and hydroxyl free radicals. The cholinesterase inhibition activity of *N. heimii* exhibited a significant IC_{50} value of 1.13 ± 0.083 µg/mL whilst weak inhibition activity against BuChE. It was concluded that *N. heimii* twigs extract contains bioactive phytochemicals compounds with antioxidant and AChE activities.

Keywords: *Neobalanocarpus heimii*; phytochemicals; antioxidant; cholinesterase inhibitory

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The Dipterocarpaceae is a very large timber tree family comprising 16 genera which are *Anisoptera*, *Balanocarpus*, *Cotylelobium*, *Dipterocarpus*, *Doona*, *Drybalanops*, *Hopea*, *Isoptera*, *Neobalanocarpus*, *Parashorea*, *Shorea*, *Stemonoporus*, *Upuna*, *Vateria* and *Vatica* and it has about 600 species, which also the most important family in economic tree in Southeast Asia [1]. *Neobalanocarpus* is a monotypic genus in the family with only a single species known as *Neobalanocarpus heimii* (King) P.S. Ashton. *N. heimii* is better known as *Chengal* in Malay and it is also common with the name '*Penak*' in Peninsular Malaysia. The species is widely distributed in Selangor, Negeri Sembilan, Western Pahang and Southern Pattani in Thailand, and can be found on low-lying flat land and on hills of up to 900 m elevation [2]. The wood of *Chengal* or *N. heimii* is resistant to fungi and termites, making it durable and the strongest timber [3]. Hence, it is commercially used for buildings, power line poles, boats, bridges and heavy construction. In addition, it is also source of a good quality resin known as dammar *penak* which is one of the finest natural dammars. The dammar *penak* is traditionally used for caulking boats, as an adhesive, and as a fuel for torches as well as commercially used in manufacture of varnishes [4, 5].

Previous research on the wood of *N. heimii* has reported 21 oligostilbenoids, including monomer, dimer, trimer and tetramer stilbenoids [6-9]. Studies have shown that resveratrol oligomers exhibited various biological activities, including antimicrobial, antifungal, antioxidant, chemopreventive agent and cytotoxicity [10, 11]. However, pharmacological studies on the oligostilbenoids isolated from *N. heimii* are limited, and there was only some research on the bioactivities of the crude extract of the plant. The wood chip extract of *N. heimii* was tested for antimicrobial activity against *Staphylococcus aureus* growth, and the result showed that methanol, ethanol, and water extracts gave the best inhibition zone with 17 ± 1.00 mm, 17 ± 0.00 mm, and 17.33 ± 0.58 mm, respectively, a slight difference between the solvent extractions [12]. The methanol extracts from the bark of *N. heimii* exhibited the highest antifungal activity against the white-rot fungus, *Pycnoporus sanguineus*, with the minimum effective amount being 100 µg, while it showed moderate antifungal activity against the brown-rot fungus, *Gloeophyllum trabeum*, and the minimum effective amount was 500 µg [13]. Besides that, a previous study found that the bark and heartwood extracts of *N. heimii* gave the highest DPPH radical scavenging activity with a percent inhibition of 93.60%

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and 83.78%, respectively [14]. Therefore, this study is focused on exploring the phytochemical screening properties and bioactivities potential of *N. heimii* crude extract, highlighting its significance for future research and applications.

EXPERIMENTAL

Chemicals and Materials

Ascorbic acid, gallic acid, Folin-Ciacalteau's reagent, quercetin, 2,2-diphenyl-1-picryl hydrazyl (DPPH), sulphuric acid, ammonium molybdate tetrahydrate, disodium hydrogen phosphate dihydrate, Ferric Reducing Antioxidant Power (FRAP) assay kit (Colorimetric), magnesium strip, hydrochloric acid (HCl), Mayer's reagent, ferric chloride ($FeCl_3$), chloroform, concentrated sulphuric acid (H_2SO_4), copper acetate solution and aqueous sodium hydroxide (NaOH) solution.

Plant Collection and Extraction

Fresh *N. heimii* twigs were collected from Hutan Simpan UiTM Jengka, Pahang. The plant sample was identified by Mr. Nik Hazlan Nik Hashim from UiTM Jengka. The voucher specimen (FSG3) was deposited at the Forest Research Institute Malaysia (FRIM) herbarium. The twigs were dried for two weeks, cut into small pieces and ground into powder. The dried powder of *N. heimii* twigs (500 g) was extracted with methanol at room temperature for 24 hours and repeated five times. The methanol extract was filtered and concentrated on a rotary evaporator under a reduced pressure of 40 ºC to give crude methanol extract.

Qualitative Phytochemical Screening

Phytochemical screening methods were carried out to confirm qualitatively the existence of main classes of compounds such as alkaloids, flavonoids, glycosides, terpenoids, saponins and tannins following the methods described by Kamarozaman and co-workers [15].

Quantitative Phytochemical Screening

Total Phenolic Content (TPC)

The total phenolic content of *N. heimii* crude extract was conducted using the method of Folin–Ciocalteu assay [16]. Briefly, 20 µL of 10 mg/mL of gallic acid standard was pipetted into a 96-well plate and conducted in triplicate. The two-fold serial dilution of the gallic acid standard was peformed, resulting in concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.039, 0.01953 and 0.0098 mg/mL, each was diluted with methanol. Moreover, 20 µL of 10 mg/mL of crude methanol extract of *N. heimii* was added to an empty column in 96-well plates. Meanwhile, methanol, which served as blank, was added into the well at 195 µL. Furthermore, 100 µL of Folin-Ciocalteu reagent was inserted into the gallic acid standard and extracted, then the plate was incubated for 5 minutes at room temperature. A 7.5% sodium carbonate with a volume of 100 µL was added into each well that contains standard and samples. Then, the plate was incubated again for 90 minutes in the dark at room temperature. Next, the absorbance was read at 740 nm by using a microplate reader. The TPC of the extract was then determined by extrapolation from a gallic acid calibration curve (0.0098 – 10 mg/mL), and the results were presented as gallic acid equivalent/g (GAE/g).

Total Flavonoid Content (TFC)

Firstly, 50 µL of 5 mg/mL of quercetin standard was pipetted into a 96-well plate, in triplicate. The two-fold serial dilution was made for the gallic acid standard at the concentration of 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.039 and 0.01953 mg/mL by diluting with deionised water. Moreover, 50 µL of 5 mg/mL of crude methanol extract of *N. heimii* was added to an empty column in the 96-well plate. The blank of deionised water was added into the well with a volume of 230 µL. A 15 µL of 5% sodium nitrite was pipetted into each well that contains standard and samples. Then, the plate was incubated at room temperature for 6 minutes. Afterwards, 15 µL of 10% aluminium chloride was added into each well that contains standard and extracts. The plate was re-incubated at room temperature for 6 minutes. Lastly, the absorbance was measured at 510 nm using a microplate reader. The total flavonoid content was calculated by extrapolation from the quercetin (0.019 – 5 mg/mL) standard curve. The results were presented as quercetin equivalent/g (QE/g).

Antioxidant Activities of Plant Extracts

Total Antioxidant Capacity (TAC)

The total antioxidant capacity was determined by the phosphomolybdate assay method reported by Chen and co-workers [17]. The phosphomolybdate dye was prepared by mixing 0.6 M H_2SO_4, 10 mL of 28 mM Na_3PO_4 together with 10 mL ammonium molybdate at the ratio of 1:1:1. Then, 40 µL of the crude extract with the initial concentration of 156 µg/mL was mixed with 260 µL previously prepared phosphomolybdate dye. Different concentrations of sample (156 µg/mL until 4.9 µg/mL) were prepared using serial dilution with methanol and mixed with 260 µL phosphomolybdate dye. For control, 40 µL of methanol was mixed with 260 µL phosphomolybdate dye. The plate was incubated for 90 minutes at 95 °C followed by 10 minutes cooling at room temperature. The absorbance was recorded at 695 nm wavelength. The TAC of several concentrations was calculated by the equation below:

$$Percentage\ Inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

The IC_{50} values were calculated by finding the 50% of inhibition of TAC using a prism. The ascorbic acid was used as standard.

DPPH Free Radical Scavenging Activity

The DPPH scavenging activity method was modified from Chen and co-workers [17]. A 0.1 mM of DPPH reagent was prepared by mixing 4 mg of DPPH powder with 100 mL of methanol. Different concentrations of the sample (156 µg/mL until 4.9 µg/mL) were prepared by using the serial dilution method. 100 µL from each concentration of sample was pipetted with 100 µL prepared DPPH solution. 100 µL methanol was mixed with 100 µL DPPH solution and labelled as control. Next, the well plate was incubated at 25 °C for 30 minutes. The absorbance was recorded at 517 nm wavelength. The percentage of DPPH radical scavenging activities of different concentrations was measured by the equation below:

$$Percentage\ Inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

The IC_{50} values were calculated by finding the 50% of inhibition of DPPH free radical scavenging activity using a prism. The ascorbic acid was used as standard.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity method was modified from Luqman and co-workers [18]. A reaction mixture of 3.75 mM deoxyribose (50 mg), 100 µM $FeCl_3$ (1.6 mg), 1 mM H_2O_2 (10 µL), 100 µM ascorbic acid (1.761 mg) was prepared and dissolved in 100 mL phosphate buffer. A 1% thiobarbituric acid (TBA) was prepared by dissolving 500 mg TBA powder with 50 mL NaOH and 2% trichloroacetic acid (TCA) was prepared by dissolving TCA powder with 50 mL distilled water. Different concentrations of the sample were prepared at a concentration ranging from 156 µg/mL to 4.9 µg/mL by using the serial dilution method with phosphate buffer. 20 µL of each sample concentration were pipetted into a 96-well plate together with 150 µL of the reaction mixture which was previously prepared. For the control, 20 µL of phosphate buffer was mixed with 150 µL of the reaction mixture. Then, the plate was incubated at 37 °C for 60 minutes and was cooled for a while at room temperature. In addition, 75 µL of 1% TBA and 75 µL of 2% TCA were added and the reaction mixture was heated at 100 °C for 15 minutes. The absorbance was read at a wavelength of 532 nm. The percentage of hydroxyl scavenging activity of different concentrations was measured by the equation below:

$$Percentage\ Inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

The IC_{50} values were calculated by finding the 50% inhibition of hydroxyl radical scavenging activity using a prism. The ascorbic acid was used as a standard.

Cholinesterase Inhibition Assays

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) Inhibition Assays

A modified Elman's method [19] was used to measure the AChE and BuChE inhibitory activities towards *N. heimii*. Firstly, 20 µL from 39.1 µg/mL of physostigmine standard and sample were added into a 96-well plate in triplicate. The serial dilution method was introduced to make different concentrations starting from 39.1 to 0 µg/mL. Then, 140 µL of 0.1 M phosphate buffer (pH 7.8) were pipetted into the standard and sample. Next, 20 µL of 0. 2U/mL AChE were added to each well and the plate was incubated at room temperature for 15 minutes. After the incubation, 10 µL of 3mM 5,5'-dithiobis-(2-nitrobenzoic acid) DTNB together with 15 mM acetylthiocholine iodide were introduced into the control and sample in the well. Each extract and standard were incubated at room temperature for 30 minutes. Afterwards, the absorbance was measured at 412 nm using a microplate reader. Then the experiments were repeated by changing the acetylthiocholine iodide and AChE solution to butyrylthiocholine iodide and BuChE solution, respectively. The percentage of AChE and BuChE inhibitory activity was calculated by the equation below [20]:

$$Percentage\ of\ inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

Statistical Analysis

All results were expressed as mean ± standard deviation with triplicate and average. The significant difference was measured at the level of $p < 0.05$. The values of IC_{50} found were used to measure statistical significance. One-way Analysis of Variance (ANOVA) using GraphPad Prism Software Version 9.5.1 was used in conducting statistical analysis.

RESULTS AND DISCUSSION

Qualitative Phytochemical Screening

The Dipterocarpaceae family is well-known for its hardwood, heavy timber plant. Furthermore, this family is also known for its phytochemical constituents, which have various advantages, particularly therapeutic properties [15]. Their crude extracts were discovered to contain the presence of alkaloids, flavonoids, glycosides, saponins, tannins, and terpenoids [21].

Table 1. Phytochemical screening from the twigs of *N. heimii.*

Plant	Screening Test					
	Shinoda Test (Flavanoid)	Mayer's Test (Alkaloids)	Foam Test (Saponins)	Ferric Chloride Test (Tannins)	Salkowski Test (Terpenoids)	Glycoside Test
N. heimii	-	+	+	+++	++	+++

Highly present (+++), moderately present (++), low amount present (+), and not present (-)

The phytochemical screening of *N. heimii* revealed the presence of several classes of compounds that might influence the antioxidant and AChE inhibition activities. The phytochemical screening from the twigs of *N. heimii* is shown in **Table 1**.

N. heimii's crude extract showed a high concentration of tannins and glycoside, a moderate composition of terpenoids and a low amount of saponins and alkaloids. However, no trace of flavonoid was detected in the *N. heimii* crude extract. Tannins are products of secondary metabolites that are widely distributed in plants. It is said to have beneficial health effects such as antioxidant properties, dressing for skin burn, antibacterial, antifungal, and diarrhoea treatment [22-24]. Meanwhile, glycosides are defined as secondary metabolites that contain a sugar moiety which attaches to non-sugar portions. Glycosides can be classified as glycone or aglycone moieties that are involved in the stimulation of the cardiac system, immune system and central nervous system [25]. Moreover, glycosides also exhibited strong antimicrobial activity [26].

Quantitative Phytochemical Screening

Total phenolic content (TPC) was calculated by extrapolation from the standard curve of gallic acid (y = 0.3297x + 0.3704, R^2 = 0.9599) as shown in **Table 2**, where it estimated by extrapolation from the gallic acid standard curve (y = 15.58x + 0.15, R2 = 0.985). The TPC of *N. heimii* is at 8.87 ± 0.075 mg GAE/g. The standard quercetin curve (y = 0.3475x + 0.2342, R^2 = 0.9671) was used to determine the TFC. The TFC of *N. heimii* is at a concentration of 0.94 ± 0.111 mg QE/g. *N. heimii* methanol crude extract showed a higher TPC value than TFC. It is suggested that the very low amount of TFC value causes negative results during phytochemical screening.

Table 2. TPC and TFC of *N. heimii.*

Crude Extract	TPC (mg GAE)/g dry extract	TFC (mg QE)/g dry extract
Neobalanocarpus heimii	8.87 ± 0.075^{a}	0.94 ± 0.111^{b}

Each value is presented as mean ± SD (n = 3). The mean with lower-case letters (a and b) are significantly ($p < 0.05$) different based on (ANOVA, Tukey-Kramer's test)

Table 3. The antioxidant activity of *N. heimii*'s crude extract presented as TAC, DPPH radical scavenging activity and hydroxyl radical scavenging activities.

Crude Extract	IC_{50} (µg/mL)		
	TAC	DPPH	Hydroxyl
N. heimii	31.17 ± 0.403	25.69 ± 0.440	24.94 ± 0.666
Standard	11.45 ± 0.714	32.36 ± 0.988	42.99 ± 0.483

Each value is presented as mean ± SD (n = 3). The mean is significantly ($p < 0.05$) different based on (ANOVA, Tukey-Kramer's test)

Antioxidant Activities of Plant Extracts

The radical scavenging activities of the standard ascorbic acid and the *N. heimii* crude extract on TAC, DPPH and hydroxyl radicals were displayed in **Table 3**. Different antioxidant activities displayed different IC_{50} values of standard. Two out of three radical scavenging activities showed *N. heimii* displayed good antioxidant activity. The IC_{50} of DPPH and hydroxyl scavenging activity are 25.69 ± 0.440 and 24.94 ± 0.666 µg/mL, respectively. The IC_{50} value of the crude extract is lower than the standard due to the high composition of phenolic compound that was shown in TPC. The high composition of phenolic compounds, tannins, glycosides as well as terpenoids in phytochemical screening of *N. heimii* acts as antioxidants by reacting with the free radicals [27]. It is suggested that the hydroxyl groups in *N. heimii* plant extract are also responsible for the acceleration of free radical scavenging, having a good impact on the IC_{50} value [28].

Cholinesterase Inhibitory Activity Assay

The AChE and BuChE inhibitory activities of *N. heimii* were evaluated according to slight modification of Ellman's method [18]. The IC_{50} (µg/mL) values for *N. heimii* were tabulated in **Table 4** in which physostigmine was used as a standard. The IC_{50} of AChE inhibition assay of *N. heimii* crude extract exhibited a significant IC_{50} value of 1.13 ± 0.083 µg/mL compared to the standard with 0.59 ± 0.156 µg/mL. However, the BuChE inhibition assay of *N. heimii* showed weak activity with an IC_{50} value of 11.81 ± 0.535 µg/mL. It was found that the crude methanolic extract of *N. heimii* possesses the ability to block AChE, which will aid in combating Alzheimer's disease (AD).

This study has shown a positive correlation between *N. heimii*'s TPC value with the results of DPPH, hydroxyl scavenging activity and AChE inhibition. It is suggested that the enzyme may possibly be inhibited by phenolic compounds as supported by a previous study [29]. Moreover, the presence of a high number of hydroxyl groups in the crude methanol extract contributed to the strong antioxidant activity as well as demonstrated good AChE inhibitory activity [30].

CONCLUSION

The phytochemical screening from the twigs of *N. heimii* revealed the presence of tannins, glycoside, terpenoids, saponins and alkaloids. Additionally, the crude methanol extract demonstrated strong activity against AChE but weak activity against BuChE with IC_{50} values of 1.126 ± 0.083 and 11.81 ± 0.535 µg/mL, respectively. Meanwhile, the antioxidant assays demonstrated strong activity against DPPH and hydroxyl radicals. It is suggested that the presence of high phenolic content as well as tannins, glycosides and terpenes influenced the antioxidant and cholinesterase inhibitory activities, which have the potential to treat AD.

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Table 4. The IC_{50} values of *Neobalanocarpus heimii*'s crude extract against AChE and BuChE.

Crude Extract	Anti-cholinesterase assays IC_{50} (µg/mL)	
	Acetylcholinesterase (AChE) inhibition assay	Butyrylcholinesterase (BuChE) inhibition assay
Neobalanocarpus heimii	1.13 ± 0.083^{a}	11.81 ± 0.535^{b}
Physostigmine (Standard)	0.59 ± 0.156^{c}	1.12 ± 0.088^{c}

The mean with lower-case letters (a, b and c) are significantly ($p < 0.05$) different based on (ANOVA, Tukey-Kramer's test)

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