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Antidiabetic, antioxidant and *in silico* studies of bacterial endosymbiont inhabiting *Nephelium lappaceum* L.

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Abstract. Endophytes, notably obtaining attention, have been abided by potential origins of bioactive metabolites. In the acquaint study, endophyte was isolated from the leaves of *Nephelium lappaceum* L. The chosen endosymbiont was identified by 16s rRNA partial genome sequencing and investigated for their antioxidant and antidiabetic activities. A preliminary phytochemical test was comported for the affirmation of phytoconstituents in endophytic crude extract (NLM). Antioxidant activities were conducted by using 2-diphenyl-1-picrylhydrazyl (DPPH) method and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method to screen the radical scavenging potential. The evaluation of antidiabetic activities was done by using α-amylase and α-glucosidase inhibition assay. Qualitative phytochemical test on NLM affirmed the presence of phenols, carbohydrates, alkaloids, flavonoids, steroids, mucilage and glycosides. *In silico* parameters were also specified for antidiabetic activities. The antioxidant assay of NLM expressed proficient antioxidant activity of IC_{50}±SEM 1.35±0.03 µg/mL and IC_{50}±SEM 1.47±0.03 µg/mL, for ABTS and DPPH respectively. Antidiabetic assay results evidenced dose dependent percentage inhibition of the enzyme. The results testified estimable inhibition of α-amylase (IC_{50}±SEM 2.549±0.08 µg/mL) and α-glucosidase inhibition (IC_{50}±SEM 2.29±0.03µg/mL) compared to the standard drug (Acarbose). *In silico* study divulged that the ellagic acid component present in the plant was responsible for antidiabetic activity. Thus, the study shows that NLM has a wellspring of natural source of antioxidants and antidiabetic agents and furtherance of studies on its mechanism is recommended to know detailed facts.

Keywords: *Nephelium lappaceum*, antioxidant, α-amylase, α-glucosidase, endophyte, *Escherichia coli*.

1. Introduction

Endophytes are notable mutualistic symbiont inhabitants within the living tissues of host plants without causing any effects. Endophytes can be distinguished as bacteria, actinomycetes or fungi. Because of the long host-parasitic relationship, endophytes can are able to produce secondary metabolites similar to that of plants. They are a wellspring of secondary metabolites, which exhibited many pharmacological activities, for instance: anticancer, antidiabetic, antifungal and antibacterial activities [1]. From a practical perspective, drug production by endophytes fermentation will provide more benefit since it will be quick, reproducible, boundless and climate/season independent. Simple to increase the microbial ability by genetic engineering and diverse cultivation condition which can produce various products. The disclosure of endophytes with a capacity to produce precisely the same active compounds produced by their host prompts another approach in active compound generation from natural product economically [1, 2]. Production of compounds can be enhanced by endophytes for bearing demands while keeping the ecosystem and biodiversity sustainable. Endophytes have pulled from into consideration in the discovery of novel bioactive compounds that can be utilized as new medications supplanting those against which pathogenic strains have quickly obtained resistance [3].

Diabetes mellitus is a metabolic disorder which happens because of impeded glucose regulation or weakened of the α-amylase enzyme. It relates to an increased risk of cardiovascular disease. Most diabetic cases included numerous genes with each being a small contributor to an increased likelihood of getting to be Type 2 diabetic and it additionally relies on the lifestyle. The medications conventionally use synthetic drugs all

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through their lifetime. Notwithstanding, long haul treatment had adverse effects, for example, oedema with gastrointestinal disorders and hyponatremia [4]. The result is inclined of today's society to favor traditional medications due to fewer side effects, sensible vale and less complex to situate in the surroundings [5].

There are unsettling impacts in antioxidant defense frameworks in diabetes mellitus [6]. As diabetic intricacies can be treated with antioxidants [7]. The commencement of the "unifying theory" that hyperglycemia-prompted oxidative stress could relate to the pathogenesis of each complexities [8].

Rambutan (*Nephelium lappaceum* L.) belongs to the Sapindaceae family is a popular tropical fruit in Southeast Asian countries namely in Indonesia, Thailand, and Malaysia. This tropical fruit is cultivated in a warm, humid, and in areas with lower evaporation rates and more rainfall. Rambutan is a red or yellow pericarp ovoid fruit which is covered with soft spines [9]. There are many traditional uses of rambutan and parts of the plant in local cultures are studied. Rambutan leaves are employed for hair care in the sense of increasing one's quality of hair [10]. Studies have reported that all parts of rambutan are useful where the leaves, rind, and seed contain anthocyanins, phenolic and flavonoids [11]. These parts are also reported to exhibit antioxidant, antibacterial, antidiabetic and cytotoxic activities [9]. The hydroxyl moiety of rambutan's phenolic compound has radical scavenging properties and reduces lipid peroxidation cell capacity. In the present study, we mainly focused on the isolated endophytes from rambutan leaves along with the screening of antioxidant and antidiabetic potentials of secondary metabolites of crude extracts of endophytes.

2. Experimental

2.1. Chemicals

2-diphenyl-1-picrylhydrazyl (DPPH), α-amylase, α-glucosidase, ascorbic acid, and gallic acid were purchased from Merck Millipore Corporation, USA. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Acarbose and Rutin were purchased from Sigma-Aldrich Corporation, USA. All the chemicals used were of analytical grade.

2.2. Plant sample collection

The plant materials, leaves of *Nephelium lappaceum* L. were collected from Baling, 09100, Kedah, Malaysia. The leaves were sampled for the study of endophytic bacterial communities. The matured and healthy plant was carefully selected for sampling, then were brought in sterile bags and processed within a few hours. The herbarium accession number of *Nephelium lappaceum* L. is AIMST/FOP/04.

2.3. Isolation of endophytic bacteria

The plant materials were rinsed gently in running water to remove superficial injury and soil particles. After proper washing, the samples were cut into small pieces. The isolation of endophytes was done according to the method described by Sadrati *et al.* (2013) with slight modifications [12, 13]. The surface sterilization was carried out by treating the plant material with 70% ethanol for 30 seconds, followed by immersion in 95% ethanol for 10 seconds and again in 5% sodium hypochlorite solution for 4 min. Subsequently, the segments were rinsed four times with sterile distilled water. The samples were cut into at least 3 to 4 mm in diameter and 0.5 to 1 cm in length. On each Petri plate, 5 to 6 segments were placed on the nutrient agar. The efficiency of the surface sterilization procedure was assessed by adding few drops of water from the last wash on the agar plate. The Petri plates were incubated at 37 ºC for 24 h to 36 h until the growth of bacteria is observed.

2.4. 16S rRNA partial gene sequencing

The PCR amplification of 16S rRNA partial gene of the selected endophyte strains was done by using the forward primer (Bakll W-F 5'- AGT TTG ATC MTG GCT CAG -3') and reverse primer (Bak-R 5'- GGA CTA CHA GGG GGG TAT CTA AT -3'). The PCR amplification was carried out in a thermocycler with the following conditions: initial denaturation at 95 ºC for 4 min, cycle denaturation at 9 ºC for 30 s, cycle annealing at 52 ºC for 30 s, cycle extension at 72 ºC for 30 s, repeat cycle steps 30 more times, final extension 72 ºC for 5 min. The sequenced DNA data were a BLAST analyzed by the NCBI database to identify the sequence similarity reported gene sequences in GenBank [14].

2.5. Bacterial endophytic crude extraction

The isolated endophyte was grown as the subculture on a nutrient agar Petri plate for 24 h under aerobic condition. The subculture was inoculated into the nutrient broth and incubated at 110 rpm on a shaker at 37 ºC for 3 to 4 days. After fermentation, the culture broth was added with brine solution and ethyl acetate solvent respectively, with the ratio of 1:3. The culture broth was then extracted. The organic phase was filtered after which anhydrous sodium sulphate was added. The ethyl acetate solvent was evaporated by rotary evaporator. The extract was dried and concentrated, then was weighed and stored at -4 ºC [15].

2.6. Phytochemical screening

NLM was used for preliminary screening of phytochemicals such as alkaloids, carbohydrates, phenols, amino acids, steroids, anthocyanins, proteins, flavonoids, saponins, mucilage, gums, glycosides and tannins using standard biochemical testing methods [16].

2.7. Antioxidant activity

DPPH free radical scavenging assay

The sample DPPH free radical scavenging capacity was assessed by using standard method [17, 18]. The sample solutions were prepared in absolute alcohol, ranging from 10 µg/mL to 1000 µg/mL. The sample (500 µL) was added with 2 µmol DPPH solution (500 µL) and kept in the dark for 20 min at room temperature. The absorbance was measured at 517 nm. Ascorbic acid was used as a reference standard.

The percentage of free radical scavenging was calculated as:

$$\%Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \quad (1)$$

where *Abs* is absorbance.

ABTS free radical cation scavenging assay

The sample capacity to scavenge, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical cation [ABTS*+] was assessed by standard method [19, 20]. 7 mM ABTS in distilled water and 2.45 mM of potassium persulfate was added. The solution was kept at room temperature in the dark for 12 to 16 h. The sample solutions were prepared in absolute alcohol, ranging from 10 μg/mL to 1000 μg/mL. The ABTS solution was added and incubated for 30 min. The absorbance was measured at 734 nm. Ascorbic acid was used as reference standard. The percentage of radical cation scavenging inhibition was calculated using Eq. 1.

2.8. In vitro antidiabetic activity

α-amylase inhibition assay

The α-amylase inhibitory activity was assayed by modifying assay [21]. The sample (500 µL) was added with 0.5 mg/mL α-amylase solution (500 µL) in 0.2 mM phosphate buffer, pH 6.9, and was incubated at 25°C for 10 min. After the pre-incubation period, 1% starch solution (500 µL) in 0.02M sodium phosphate buffer, pH 6.9, was added and incubated for 10 min at 25 °C. After the addition of 1 mL of dinitrosalicylic acid color reagent, the solutions were kept for incubation in boiling water for 5 min subsequently cooled to room temperature. The absorbance was measured at 540 nm. Acarbose was used as a reference standard. The percentage of inhibition was calculated using Eq. 1.

α-glucosidase inhibition assay

The α-glucosidase inhibitory activity was assayed by modifying assay [22]. The sample (1 mL) and 2% M/V sucrose (1 mL) in Tris buffer, pH 8, were incubated for 5 min at 37 °C. Then, 1 U/mL α-glucosidase (1 mL) was added and incubated at 37 °C for 10 min. The reaction was arrested by heating in boiling water for 2 min. The glucose content was measured by the glucose peroxidase method. Acarbose was used as a reference standard. The percentage of inhibition was calculated using Eq. 1.

2.9. Statistical analysis

The data were computed as mean ± standard error mean (SEM) and conveyed in triplicates ($n = 3$) throughout the experimental procedures. The IC_{50} values for both antioxidant and antidiabetic studies are calculated using the Graph Pad Prism Software (Version 5) by non- linear regression graph plotted between the percentage of enzyme inhibition (x axis) against concentrations (y axis) for antidiabetic assay and between percentage of radical scavenging (x axis) against concentration (y axis) for antioxidant assay.

2.10. In silico studies

One of the important secondary metabolites commonly found in this plant is ellagic acid. The ellagic acid is dilactone of hexahydroxydiphenic acid (Figure 1). Due to different medicinal properties of ellagic acid, they have enabled the researcher to obtain its potential health benefits [23]. To illustrate the antidiabetic nature of the Rambutan plant product an *in silico* case study was performed. The binding affinity of the ellagic acid with two receptors, α-amylase and α-glucosidase was evaluated by molecular docking study by using HEX 8.0 tool [24].

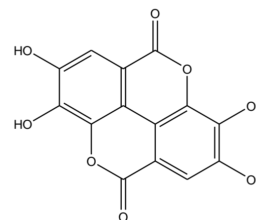

Figure 1. Structure of dilactone of hexahydroxydiphenic acid.

3. Results and discussion

Endophytic bacteria habitats in various plant tissues are relatively unstudied and are known to have a wellspring of novel natural products to be utilized as part of industry, agriculture and medicine. The significance of endophytes has become apparent recently when it was demonstrated that it plays a part as protecting the host plant against ailments and insects [25]. In this study, endophytes were isolated from the leaves of *Nephelium lappaceum* L. The surface sterilization of the excised tissue was carried out to ensure the expulsion of surface microbial flora especially epiphytes. The immersion of the tissue in sodium hypochlorite and ethanol demonstrated significant accomplishment in various studies to isolate the endophytes [26]. The small pieces of the plant tissue (leaves) under aseptic conditions were moved to the isolation media. The endophytes were chosen, and the cultures were selected by a repeated sub culturing on Lysogeny broth (LB) agar and characterized by16S rRNA gene sequencing.

The genome sequence data obtained from 16S rRNA gene, partial sequencing was used in the identification of bacterial endophyte. The 16S rRNA sequence nucleotide blast analysis reveals the identities of the sample based on his analysis from Megablast (highly similar sequences) output. Based on the 16S rRNA sequence nucleotide blast analysis, the closest hit was treated as the identity of the respective endophytes sample. Based on the results obtain through 16S rRNA sequence nucleotide blast analysis, the endophyte identified as *Escherichia coli*. The 16S rRNA gene fragment nucleotide sequences have been submitted to the GenBank/DDBJ/EMBL under accession number of MF615205.

On performing preliminary qualitative phytochemical analysis using standard procedures [27], NLM reported to have various phytoconstituents such as alkaloids, flavonoids, steroids, mucilage, proteins, reducing sugar and phenols.

Table 1. α-amylase and α-glucosidase enzyme inhibition by NLM and Acarbose

Concen-tration (µg/mL)	α- amylase		α- glucosidase	
	Acarbose	NLM	Acarbose	NLM
100	39.39	20	40	34.96
200	57.58	41.21	52	48.72
400	54.55	50.30	68.8	65.44
800	69.70	58.48	80.8	78.08
1000	93.94	84.55	90.4	88.48
IC_{50}±SEM	2.25±0.15	2.549±0.08	2.22±0.04	2.29±0.03

The crude extract of endophyte is subjected to *in vitro* antioxidant assay and the scavenging effect of free radicals was screened by using both ABTS and DPPH methods. Ascorbic acid was used as a standard drug for both assays. The sample and standard are diluted in absolute alcohol at the concentrations of 10, 25, 50, 100, 250, 500 and 1000 µg/mL. As depicted in Figure 2(a) and 2(b), the scavenging effect of highest concentration, 1000 µg/mL of NLM and ascorbic acid showed 92.1% (IC_{50}±SEM 1.35±0.03 µg/mL) and 96.7% (IC_{50}±SEM 1.07±0.09 µg/mL) respectively in ABTS assay. In DPPH assay, the scavenging effect of highest concentration, 1000 µg/mL of NLM and standard showed 89.5% (IC_{50}±SEM 1.47±0.03 µg/mL) and 94.38% (IC_{50}±SEM 1.37±0.09 µg/mL) respectively.

Plants have different free radicals scavenging molecules that are abundant in antioxidant activity [28]. Most plants are wellspring of phenolics, flavanols, carotenoids, vitamin E and ascorbic acid, which have the scavenging capability in the human system [29, 30]. Various polyphenolic constituents acquired from plants are more practical antioxidants and as needs it may be add to defensive effects *in vivo*. As indicated by past investigations [31], phytoconstituents with potent antioxidants have been known to be great enzyme inhibitors, hyperglycemia regulators and other diabetic intricacies resulting from oxidative stress.

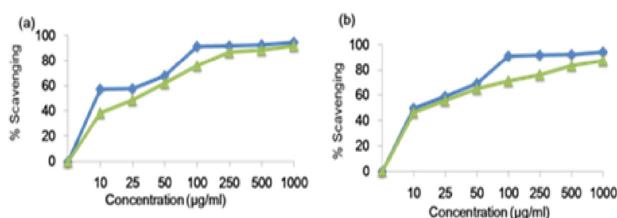

Figure 2 (a): ABTS radical scavenging assay of ascorbic acid (blue color) and NLM (green color); **2 (b):** DPPH radical scavenging assay of ascorbic acid (blue color) and NLM (green color)**.**

Compared with the standard Acarbose, the extract at maximum concentration showed significant α amylase inhibition activity of 84.55% (IC_{50}±SEM 2.549±0.08 µg/mL) and 93.94 (IC_{50}± SEM 2.251±0.15 µg/mL) for NLM and Acarbose respectively as appeared in Table 1. For α-glucosidase inhibition assay, the extract demonstrated that the concentration is dependent to percentage of α-glucosidase inhibition. The concentration needed for 50% inhibition (IC_{50}±SEM) was found to be 2.29±0.03 µg/mL for NLM. The IC_{50}±SEM of Acarbose for α-glucosidase inhibitory activity was found in 2.22±0.04 µg/mL as shown in Table 1.

The pancreatic α-amylase is one of the fundamental enzymes in the human stomach, as it breaks down starch into oligosaccharides and disaccharides. The glucose product is later ingested into the blood circulation. The α-amylase inhibition would reduce the starch breakdown in the intestinal tract. In this way, the postprandial hyperglycemia level may decrease [32]. The enzyme inhibitory effect suggests that the active components (secondary metabolites) competes with the substrate for binding to the active site of the enzyme, thus preventing the breakdown of oligosaccharides to disaccharide and resulting from the anomalous bacterial fermentation of undigested carbohydrates in the colon and therefore mild enzyme activity is desirable [33].

Ellagic acid, which was found as important secondary metabolites of rambutan, and was chosen as a chemical constituent for the present *in silico* docking study [22]. One of the important secondary metabolites commonly found in this plant is ellagic acid. The docking conformation of the ellagic acid with the different receptors of α-amylase and α-glucosidase was depicted in Figure 3(a) and 3(b).

Several researches on ellagic acid have been done and specifically Chao *et al.* (2009) reported about the supplement of both ellagic acid can prevent diabetic cardiomyopathy [34]. Hex 8.0.0 is a popular protein docking program that contains an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein and ligand molecules. This program is capable of calculating the protein-ligand docking, assuming the ligand is rigid, also it superposes pairs of molecules using only knowledge of their 3D shapes. It uses Spherical Polar Fourier (SPF) correlations to accelerate the calculations and its one of the few docking programs which has built in graphics to view the results. During the docking process the default settings of the parameter were used in Hex 8.0 tools as shown in Table 2.

Table 2. Parameters set for the docking process.

No.	Parameters	Set conditions
1	Correlation type	Shape only
2	FFT Mode	3D fast lite
3	Grid dimension	0.6
4	Receptor range	180
5	Ligand range	180
6	Twist range	360
7	Distance range	40

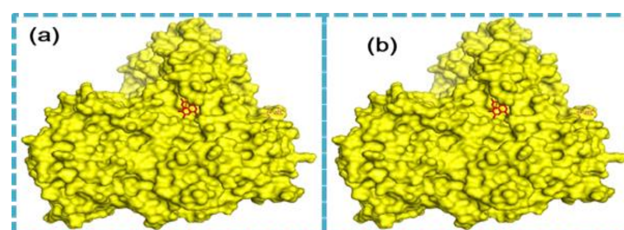

Figure 3(a): The docking conformation of the ellagic acid (red stick) with the different receptors (yellow in color) of α-amylase; **3(b):** The docking conformation of the ellagic acid (red stick) with the different receptors (yellow in color) of α-glucosidase.

For docking study, the crystal structure was obtained from the protein data bank (PDB) available at www. rcsb.org/pdb. The PDB id 3l4w receptor was used for α-glucosidase and 3ole was used as the receptor for α-amylase. Similarly, the ellagic acid structure was obtained from the pdb.

The docking energy (*E* values) was computed as -263.10 in case of α-glucosidase and -278.79 for α-amylase which is an indication of their interaction. The

result showed the *E* value obtained from the docking process is more compatible with the receptor that evaluates the antidiabetic nature of the ellagic acid. However, these molecules need to be further screened extensively through *in vitro* and *in vivo* experimentation before reaching any deceive conclusion.

4. Conclusions

The present study presumes that the presence of bioactive compound in the crude extract from the endophyte bacteria *Escherichia coli,* present in the leaves of *Nephelium lappaceum* L. exhibited promising antioxidant and antidiabetic properties. It merits saying that the *Nephelium lappaceum* L. which harbor the endophytes of the present study has been generally utilized in folklore medicine. Endophytes within these plants hold extraordinary potential in producing bioactive compounds. *In silico* study revealed that the ellagic acid component present in the secondary metabolite of the endophyte might be responsible for antidiabetic activity. Additional research like purification and fractionation of the active compounds in the crude extract of the endophyte and their structural elucidation will uncover the obscure compounds in the isolates and helps in the development of future drugs.

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Conflict of interest

All the authors ensure that there is no conflict of interest regarding authorship, or any other matters pertaining to this manuscript.

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