THE PRODUCTION OF EXO- AND ENDO- β- GLUCAN FROM MALAYSIAN *Ganoderma lucidum* IN A REPEATED BATCH FERMENTATION AND ITS ROLE AS α-GLUCOSIDASE INHIBITOR

BY

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ABSTRACT

Polysaccharides extracted from *Ganoderma lucidum* (GL) were reported previously as promising anti-diabetic drugs. They have shown inhibition of glucosidase enzyme, one of the primary targets for type 2 diabetes mellitus (T2DM) treatment. This study aims to identify the roles of EPS and ENS extracted from GL in inhibiting the α-glucosidase enzyme. Upscale production of GL was done using a 10L bioreactor. The zebrafish embryo toxicity test was carried out based on OECD guidelines. For diabetes induction, the adult zebrafish (3–4 months of age) were overfed and induced with three doses of 350 mg/kg streptozotocin (STZ) by intraperitoneal injection (IP) on three different days (Day 1, 3, and 5). Oral sucrose tolerance test (OSTT) and anti-diabetic activity of exo-β-glucan (EPS-BG) and endo-β-glucan (ENS-BG) were evaluated (Day 7) using the developed model (n = 15). This study showed both EPS-BG (IC_{50} = 0.1575 mg/mL) and ENS-BG (IC_{50} = 0.3479 mg/mL) demonstrated a strong inhibition towards α-glucosidase activity similar to the clinically approved α-glucosidase inhibitor, acarbose (IC_{50} = 0.8107 mg/mL). ENS is non-toxic towards zebrafish embryos with LC_{50} of 0.92 mg/mL and showed no significant changes in zebrafish embryo hatching and normal heart rate as compared to untreated embryos (161 beats/min). Teratogenic effects of ENS (<1.0 mg/mL) on zebrafish embryonic development were not observed. The DM model of zebrafish were acquired after the third dose of STZ with a fasting BGL of 8.98 ± 0.28 mmol/L compared to the normal healthy group (4.23 ± 0.62 mmol/L). The BGL of DM zebrafish after 30 minutes treated with EPS-BG and ENS-BG showed a significant reduction. Both EPS-BG and ENS-BG significantly reduced DM zebrafish's peak blood glucose and area under the curve in OSTT. Hence, from the study, GL mycelial pellets withstood seven cycles of long fermentation conditions and possessed anti-diabetic properties, which suits large-scale natural drug fermentation. EPS-BG and ENS-BG extracted from GL showed promising inhibition of the α-glucosidase enzyme and are considered non-toxic in ZE. Moreover, EPS-BG and ENS-BG reduced blood glucose levels and inhibited hyperglycaemia in DM zebrafish.

ملخص البحث

بينت دراسات سابقة أنَّ السكريات المستخرجة من فطر الريشي (GL) تعدُ أدوية واعدة لمعالجة مرض السكري. فقد أظهرت قدرتها على تثبيط إنزيم الجلوكوزيداز، وهو أحد الطرق الرئيسية لعلاج مرض السكري من النوع ٢ .تهدف هذه الدراسة إلى تحديد دور (EPS) و(ENS) المستخلصان من (GL) في تثبيط إنزيم (α-glucosidase) .تم تحفيز إنتاج (GL) باستخدام مفاعل حيوي سعة ١٠ لتر. تم إجراء اختبار سمية جنين السمك الزرد بإرشادات من منظمة التعاون الاقتصادي والتنمية (OECD). لتحفيز الإصابة بمرض السكري، تُطعم أسماك الزرد البالغة (من 3 إلى 4 أشهر) حتى التخمة ثم تحقن بثلاث جرعات من ٣٥٠ مجم /كجم من الستربتوزوتوسين (STZ) عن طريق الحقن داخل الصفاق (IP) في ثلاثة أيام مختلفة (اليوم ١ و٣ و٥). قيم اختبار تحمّل السكروز الفموي (OSTT) والنشاط المضاد لمرض السكري لبيتا دي جلوكان الخارجيّ وبيتا دي جلوكان الداخلي كانت مرتفعة (يوم ٧) باستخدام النّموذج المطور($n = 15$) . أظهرت هذه الدراسة أن كل من (EPS-BG) ($IC_{50} = 0.1575$ mg/ml) و (ENS-BG) ($IC_{50} = 0.3479$ mg/ml) يعد مثبطاً قويًا تجاه نشاط (α-glucosidase) مشابه لـ (α-glucosidase inhibitor) المعتمد سريريًا، acarbose ($IC_{50} = 0.8107$ mg / mL). (ENS) غير سام تجاه أجنة السّمك الزرد مع (LC_{50}) من (0.92 mg/ml) ولا يظهر أي تغيرات مهمة في فقس أجنة سّمك الزرد (ZE) ومعدل ضربات قلب طبيعية مقارنة بالأجنة غير المعالجة (161 beats/min). لم يتم ملاحظة تأثيرات متعلقة بالتشوهات الجنينية ENS(1.0 mg/ml>) على التطور الجنيني لأسماك الزرد. تم الحصول على نموذج (DM) من سمك الزرد بعد الجرعة الثالثة من (STZ) مع صيام (BGL) (8.98 ± 0.28 mmol/L) مقارنة بالمجموعة الصحية العادية (4.23 ± 0.62 mmol/L). أظهر (BGL) لأسماك الزرد (DM)انخفاضًا كبيرًا بعد 30 دقيقة من المعالجة بـ (EPS-BG) و (ENS-BG). خفض كل من (EPS-BG) و (ENS-BG) بشكل كبير ذروة جلوكوز الدم في أسماك الزرد (DM)والمنطقة الواقعة تحت المنحنى في (OSTT). في الدراسة، صمدت كرات الأُفطوري (GL mycelial pellets) سبع دورات من حالات التخمر الطويلة وامتلكت خصائص مضادة لمرض السكري، والتي تناسب تخمر الأدوية الطبيعية على نطاق واسع. أظهر (EPS-BG) و (ENS-BG) المستخلصان من (GL) تثبيطًا مباشراً لإنزيم (α-glucosidase) ويعتبران غير سامين في استخدامهما لأسماك الزرد (ZE). بالإضافة إلى أنَّ (EPS-BG) و (ENS-BG) استطاعا تخفيض مستويات الجلوكوز في الدم وتثبيط ارتفاع السكر في الدم في سمك الزرد (DM) .

APPROVAL PAGE

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LIST OF SYMBOLS

°C	Degree Celsius
g	Grams
h	Hour
K_m	Michaelis-Menten constant
L	Liter
mg	Milligrams
min	Minutes
mL	Milliliter
rpm	Revolutions per minute
V_{max}	Maximum velocity
v/v	Volume per volume
VVM	Volume of air per volume of culture per minute
α	Alpha
β	Beta
µg	Micrograms
µm	Micrometers (microns)

LIST OF ABBREVIATION

AGI	α-glucosidase inhibitor
BGL	Blood glucose level
BRR	Broth replacement ratio
BRTP	Broth replacement time point
DM	Diabetes mellitus
ENS	Endopolysaccharide
ENS-BG	Endo-β-Glucan
EPS	Exopolysaccharide
EPS-BG	Exo-β-Glucan
FTIR	Fourier Transform Infrared Spectroscopy
GL	*Ganoderma lucidum*
Hpf	Hour post-fertilization
IP	Intraperitoneal injection
NMR	Nuclear Magnetic Resonance
OECD	Organization for Economic Cooperation and Development
OSTT	Oral sucrose tolerance test
RBF	Repeated batch fermentation
SLF	Submerged liquid fermentation
STZ	Streptozotocin
ZE	Zebrafish embryo
ZFET	Zebrafish embryo toxicity test

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Ganoderma lucidum (G. lucidum) is one of the broadly utilized species in the biochemical and pharmaceutical fields. The research grew rapidly on the metabolite or complex produced, for example, ganoderic acid and polysaccharide for their medicinal convenience and was considered as a "remedy that could resuscitate the dead" (R. Ahmad et al., 2021; Baby et al., 2015; Du et al., 2021; Zhou et al., 2015). These days, Ganoderma has been utilized to prevent and treat numerous kinds of disorders and well-being items that are accepted to have anti-cancer properties, anti-ageing, and are hostile to microbial or against viral capacities. These products of *Ganoderma* are accessible, particularly in East Asia and the USA (Paterson, 2006; Seweryn et al., 2021). The amount of publication that discussed on the separation, bioactivity, and production of bioactive auxiliary metabolites of *G. lucidum* has expanded due to their extraordinary healthy benefit, as well as their bottomless and interesting optional metabolites as a promising potential library for new medication disclosure.

Two methods used to cultivate this mushroom are solid-state fermentation (SSF) and submerged liquid fermentation (SLF). SSF takes months, to develop this mushroom for their fruiting body generation. In contrast, other options are SLF, which can cut the period of getting their liquid mycelium biomass and polysaccharides to only several weeks (Cho et al., 2015). However, according to previous research, Repeated batch fermentation (RBF) is by all accounts the most encouraging liquid cultivation method as it can produce more biomass in a shorter time as compared to other type of fermentation.

RBF is an adjustment of a current method where the medium or a specific amount of a medium is expelled. Then, the new medium is presented intermittently or more than once without changing the current culture. The RBF appeared to be powerful

in enhancing microbial productivity (Birhanli & Yesilada, 2010; Wan Mohtar et al., 2016). Effective and efficient production of bioactive compounds such as polysaccharides and ganoderic acid from medicinal fungi is vital to meet the demands for various applications in the pharmaceutical industry and the cosmetics and personal care industries (Ahmad Usuldin et al., 2021).

In this study, our focus was on the production of exopolysaccharide (EPS) and endopolysaccharide (ENS) from Malaysian *Ganoderma lucidum* for the regulation of postprandial hyperglycaemia, focusing on inhibiting the α-glucosidase enzyme mechanism. α-glucosidase is an enzyme that catalyses the hydrolysis of starch to simple sugars for intestinal absorption, and the α-glucosidase inhibitor (AGI) such as acarbose and miglitol are particularly advantageous for reducing postprandial blood glucose levels by delaying the digestion of carbohydrates into glucose. The synthetic antidiabetic drugs were reported to have many side effects on diabetic patients' health. The most often reported adverse effects of these medications are gastrointestinal problems. Acarbose lowers blood glucose by slowing down the carbohydrate's digestion. However, in the colon, the bacteria that degraded the undigested carbohydrates cause excessive gas formation, resulting in flatulence, diarrhoea, and abdominal pain (Akmal & Wadhwa, 2021). Other than that, these synthetic drugs also pose a distinct adverse effect on diabetic patients, including hypersensitivity reactions, lactic acidosis, liver failure, acute pancreatitis, and weight gain (Chaudhury et al., 2017). The limitation of these drugs concerning the significant side effects on the patient has paved the way to the search for alternative solutions, considering the use of natural products derived from medicinal mushrooms as a promising compound to treat diabetes.

This study explores the potential of polysaccharides extracted from Malaysian *Ganoderma lucidum* (GL) as a natural α-glucosidase inhibitor. Reports have been made regarding the use of substances and extracts derived from GL as antidiabetic substances. GL has been shown previously to exert an antidiabetic effect; however, to date, an in-depth explanation of the mechanisms underlying its antidiabetic effect is still scarce (M. Liu et al., 2018).

1.2 PROBLEM STATEMENT

The conventional method of producing *Ganoderma lucidum*, which is solid-state fermentation (SLF), requires a time-consuming and lengthy incubation period. It takes several months for fruiting bodies to appear and extremely susceptible to contamination during that time. As a result, the repeated batch fermentation technique was used as an alternate method of producing an effective and efficient number of bioactive compounds in a shorter time in meeting the demands for a broad range of applications across sectors.

Current α-glucosidase inhibitors that have been utilized to treat type 2 diabetes (T2D) cause adverse side effects, including diarrhoea, abdominal bloating, flatulence, and distention. Thus, an urgent need to search for natural α-glucosidase inhibitors with no or fewer side effects. The polysaccharides extracted from *Ganoderma lucidum* have been proven to exhibit antidiabetic effects and play a role in blood glucose regulation. Hence, this study attempted to explore the *in vitro* α-glucosidase inhibition activity of EPS-BG and ENS-BG from the mycelium of Malaysian *Ganoderma lucidum* strain QRS 5120. Furthermore, the *in vivo* effect of EPS-BG and ENS-BG in reducing the blood glucose level was analysed in induced diabetic adult zebrafish models, potentiating the use of these compounds as natural drugs in the treatment of type 2 diabetes.

1.3 RESEARCH OBJECTIVES

Generally, this study aims to identify the roles of EPS and ENS extracted from *Ganoderma lucidum* in inhibiting the α-glucosidase enzyme with a specific objective listed below.

1. To identify the efficiency and morphological changes of Malaysian *Ganoderma lucidum* mycelium in a repeated batch fermentation for the production of EPS and ENS.

2. To demonstrate the *in vitro* natural α-glucosidase inhibitory reaction of Exo- and Endo-β-glucan from Malaysian *Ganoderma lucidum*.

3. To evaluate the *in vivo* effect of Exo- and Endo-β-glucan in reducing the blood glucose level of induced diabetic adult zebrafish.

1.4 HYPOTHESIS

1. RBF will improve the exopolysaccharide (EPS) and endopolysaccharide (ENS) production of *Ganoderma lucidum* strain QRS5120.

2. The Exo- and Endo-β-glucan from *Ganoderma lucidum* strain QRS5120 inhibits the *in-vitro* α-glucosidase enzyme activities.

3. The Exo- and Endo-β-glucan from *Ganoderma lucidum* strain QRS5120 lowered the blood glucose level in induced diabetic adult zebrafish.

1.5 SIGNIFICANCE OF THE STUDY

The ideal cultivation factors and conditions of RBF for *Ganoderma lucidum* strain QRS5120 can be used as a blueprint for an upscale bioreactor application in future use. The natural α-glucosidase inhibitory reaction of Exo- and Endo-β-glucan from Malaysian *Ganoderma lucidum* is crucial for developing new antidiabetic drugs (α-glucosidase inhibitors) from a natural source which is a safe way to control Type 2 Diabetes (T2D) and effective compared to the conventional medicines.

1.6 CONCEPTUAL FRAMEWORK

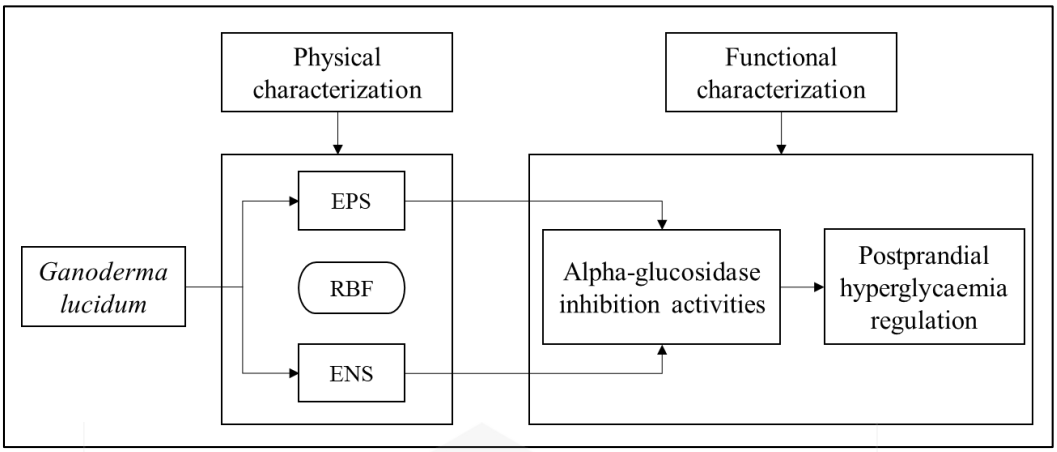

Figure 1.1 Conceptual framework

CHAPTER TWO

LITERATURE REVIEW

2.1 *Ganoderma lucidum*

In recent years, medicinal mushrooms have been extensively researched for their potential to alleviate disease, and they have been ingested throughout history, particularly in Asian countries (Seweryn et al., 2021). *Ganoderma lucidum* (GL) is a genus of Ganoderma from the Ganodermataceae family of Polyporales order that is included in the class of Agaricomycete from phylum Basidiomycota. Before, this mushroom is widely known and been recognised as 'Lingzhi' in China, but researchers recently discovered that 'Lingzhi' was different from the GL based on both morphological and molecular data (Dai et al., 2017). Lingzhi has traditionally been regarded as a magical herb as well as an auspicious emblem in Chinese culture (Z. Lin, 2019).

Ganoderma lucidum has been widely used for traditional medicine for over two thousand years and has proven to possess numerous therapeutic properties, including anti-cancer (Syairah et al., 2018; J. J. Zhang et al., 2021), anti-hyperglycaemic (Alzahrani & Pinto, 2021; Lee et al., 2020; Sarnthima et al., 2017; J. J. Zhang et al., 2021), antiviral (Wu et al., 2019), antibacterial (Savin et al., 2020; C. Wang et al., 2019), cardioprotective (Chan et al., 2021), and wound healing properties (Yin et al., 2019).

2.1.1 *Ganoderma lucidum* Strain QRS 5120

Ganoderma lucidum strain QRS 5120 was morphologically identified and sequenced using phylogenetic software (Supramani, Ahmad, Ilham, et al., 2019). The study showed that the *Ganoderma lucidum* strain QRS 5120 was found to yield 637 base pairs, which was comparable to other Ganoderma species. The discovery of this novel strain sparked a flurry of investigations into a variety of fields and aspects. Supramani, Ahmad, & Wan-Mohtar, (2019) studied the antimicrobial effect of beta-glucan from *G. lucidum* QRS 5120 mycelium against four species of pathogenic bacteria (*S.*