IDENTIFICATION OF α-GLUCOSIDASE INHIBITORS FROM BLACK GARLIC

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กระเทียมดำเป็นผลิตภัณฑ์อาหารเพื่อสุขภาพที่เติบโตเร็วที่สุดชนิดหนึ่งในตลาดอาหารเพื่อสุขภาพในปัจจุบัน โดยเฉพาะอย่างยิ่งในทวีปเอเชีย กระเทียมดำนี้ได้มาจากการบ่มหัวกระเทียมสดภายใต้อุณหภูมิและความชื้นที่สูงเป็นเวลาหลายวัน โดยกระเทียมดำที่ได้เมื่อเทียบกับกระเทียมสดแล้ว มีลักษณะหลายอย่างที่แตกต่างออกไป เช่น สี กลิ่น รสชาติ เนื้อสัมผัส สารอาหาร องค์ประกอบทางเคมี รวมทั้งการออกฤทธิ์ทางชีวภาพและคุณสมบัติทางยาหลายด้าน หนึ่งในนั้นคือการต้านเบาหวาน แต่ยังไม่มีรายงานว่าสารใดที่เป็นตัวออกฤทธิ์ในการต้านเบาหวาน ในงานวิจัยชิ้นนี้จึงได้ใช้ฤทธิ์ในการต้านอัลฟากลูโคซิเดสเป็นแนวทางในการแยกสารสำคัญ และได้สารสำคัญสี่ตัวคือ 5-(hydroxymethyl)furan-2-carbaldehyde (5-HMF), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFCA), 4-(hydroxymethyl)phenol และ 2-Deoxy-ribono-1,4 lactone เมื่อทดสอบประสิทธิภาพในการยับยั้งการทำงานของเอนไซม์อัลฟากลูโคซิเดส พบว่า HMFCA, 4-(hydroxymethyl)phenol และ 2-Deoxy-ribono-1,4 lactone มีฤทธิ์ต้านการทำงานของเอนไซม์ดังกล่าว สำหรับมอลเทสมีค่า IC50 คือ 18.83±0.91, 23.70±1.52 และ 9.26±0.31 mM ตามลำดับ ในขณะที่ซูเครสเป็น 17.42±0.84, 6.78±0.17 mM ตามลำดับ และผลการศึกษากลไกการออกฤทธิ์พบว่า HMFCA และ 4-(hydroxymethyl)phenol ยับยั้งทั้งมอลเทสและซูเครสโดยกลไกแบบ uncompetitive ส่วน 2-Deoxy-ribono-1,4 lactone ยับยั้งมอสเทสผ่านกลไก competitive

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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Black garlic has emerged as one of the fastest-growing health food products on the market. It is processed by incubating fresh garlic under high temperature and high humidity conditions. The process causes the changes in characteristics of black garlic such as texture, flavor, nutrient contents, chemical composition and it pharmacological properties, especially antidiabetic activity. In the present investigation, we applied α-glucosidase inhibition guided isolation to identify active compounds. Four compounds named 5-hydroxymethylfurfural (5-HMF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 4-(hydroxymethyl)phenol and 2-deoxy-ribono-1,4 lactone were isolated. On evaluation against α-glucosidase, HMFCA, 4-(hydroxymethyl)phenol, and 2-deoxy-ribono-1,4 lactone were active against maltase with IC_{50} values of 18.83 ±0.91, 23.70 ±1.52, and 9.26±0.31 mM respectively. Meanwhile, HMFCA and 4-(hydroxymethyl)phenol were also active against sucrase with IC_{50} values of 17.42 ±0.84 and 6.78 ±0.17mM, respectively. The kinetic study suggested that HMFCA and 4-(hydroxymethyl)phenol could inhibit the maltase and sucrase by uncompetitive manner. On the other hand, 2-deoxy-ribono-1,4 lactone was identified as a non-competitive inhibitor against maltase.

Field of Study: Biotechnology Student's Signature .....

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Pattraporn Tantiwatcharachai

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Introduction

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1.1.1 Diabetes mellitus

Diabetes is a chronic non-communicable disease that is found in a large population and there is an increasing trend [1]. It is a disease associated with metabolic disorders caused by abnormal secretion or dysfunction of insulin. There are two type of diabetes, Type 1 the body doesn't make insulin it immune system attacks and destroys insulin-producing cells in the pancreas so the patient must take artificial insulin everyday to stay alive. Type 2 the body is still able to make insulin, but the cells become resistant to insulin [2]. This type is the most common type of diabetes (Figure 1). Long-term chronically high blood sugar levels of this disease will relate to damage, dysfunctions, and failure of various organs.

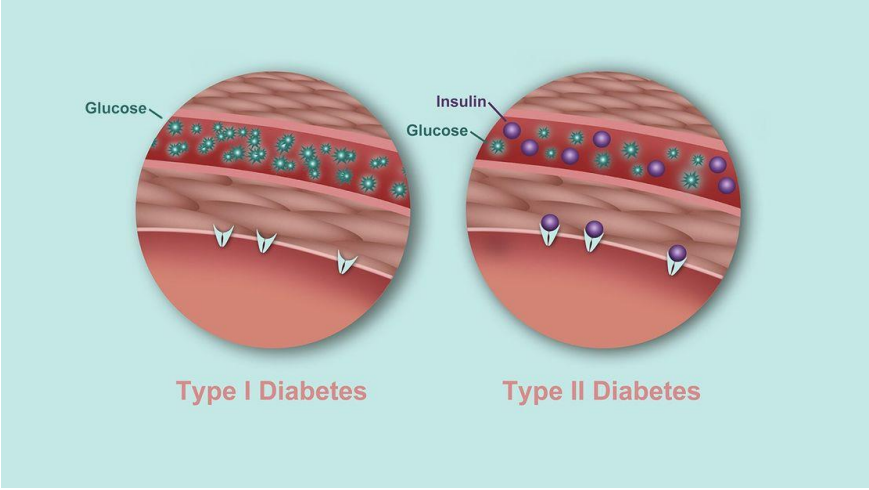

Figure 1 Type of diabetes mellitus

(https://www.everydayhealth.com/diabetes/difference-between-type-1-type-2-diabetes/)

Failure to control blood sugar levels can lead to diabetes complications like cardiovascular disease, hyperlipidemia and retinopathy [3]. Current treatments are either insulin injection or a synthetic drug, depending on the type of disease. However, prolonged use of synthetic drugs such as metformin, sulfonylurea derivatives and meglitinides (Figure 2) can cause side effects in some patients like vomiting, gastrointestinal side effects, hypoglycemia and weight gain [4] [5] [6]. Therefore, many studies have attempted to introduce natural products with sugar-regulating properties as a substitute for synthetic drugs. In regulating blood sugar levels, it is primarily related to suppress carbohydrate metabolism pathways which have many enzymes involved such as dipeptidylpeptidase-4 and α-glucosidase.

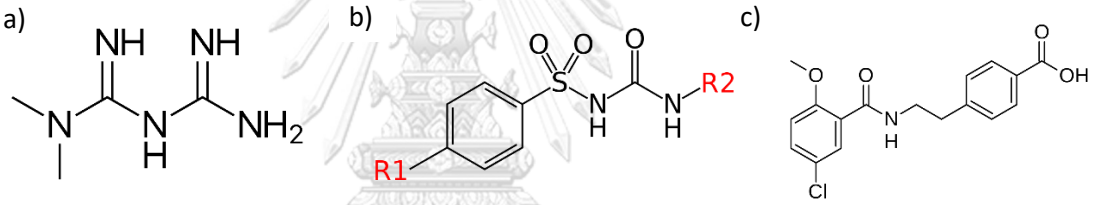

Figure 2 Chemical structures of antidiabetic drugs (a) metformin (b) sulfonylurea derivatives and (c) meglitinide

1.1.2 α-Glucosidase

Alpha glucosidase is a group of enzymes located in the brush border surface of small intestine. It responsible for the digestion of carbohydrate macromolecules like starch and polysaccharides down to a monosaccharide glucose that can be absorbed by the small intestine into the bloodstream [7] [8]. The high rate of polysaccharide digestion results in rapid increase in postprandial blood glucose level (Figure 3 and 4) Therefore, if the enzyme activity can be reduced, it will lead to a decrease in glucose absorption, thus resulting in lower glucose levels in the bloodstream after meals(Figure 5) [9]. Thus, the use of alpha-glucosidase inhibitor (AGI) is an interesting hyperglycemia regulator.

Currently, there are strong potential alpha-glucosidase inhibitors that are used as commercial drugs, like acarbose, miglitol, and voglibose [10].

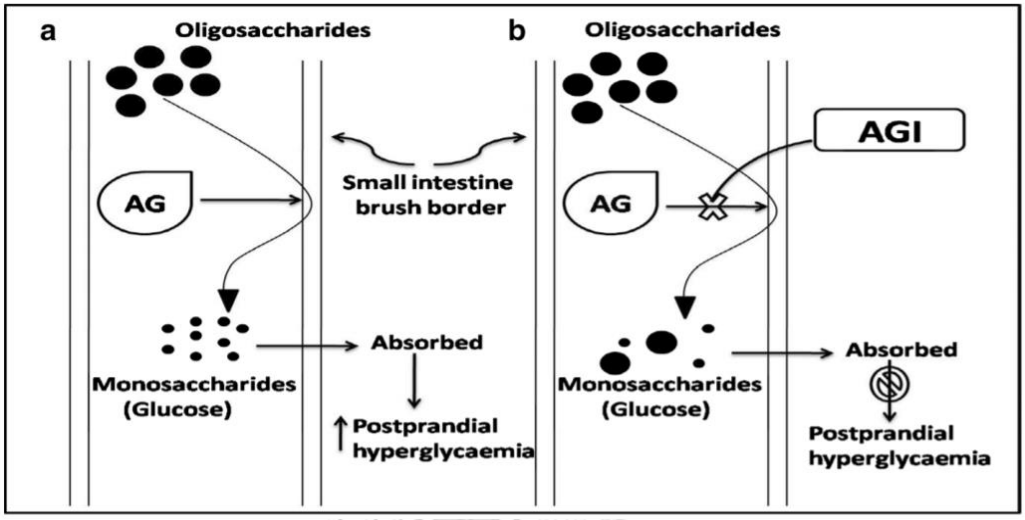

Figure 3 α-glucosidase function

(https://www.researchgate.net/figure/Mechanism-of-action-of-alpha-glucosidase-inhibitors_fig2_279991207)

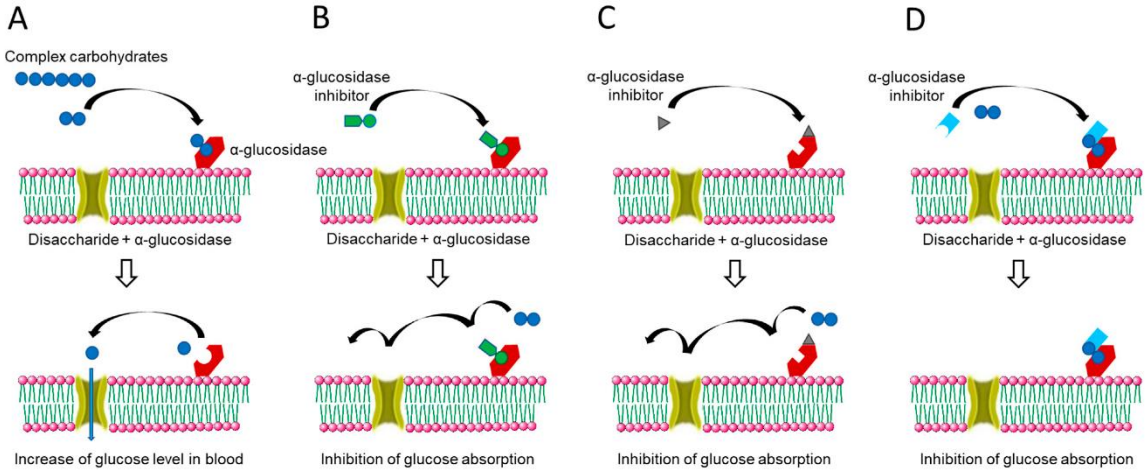

Figure 4 Mechanism of α-glucosidase inhibitors

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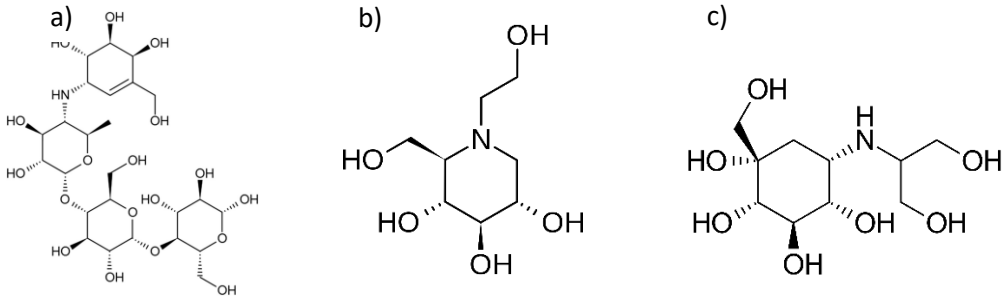


Figure 5 Chemical structures of commercialized α-glucosidase inhibitors (a) acarbose (b) miglitol (c) voglibose

1.2 Black garlic

1.2.1 General characteristics

Black garlic is a rapidly growing health product in the global health food market. Nowadays, it can be seen in various media such as food programs, animation, and TV series. In Asian countries, black garlic is very popular and has a long history of consumption, including in Thailand, Korea, Japan, and China [11] [12]. Black garlic is obtained from fresh garlic by thermal incubation (60-80 C°) of whole bulb of fresh garlic over several weeks under controlled humidity [13] [14]. The idea of producing black garlic is originally aimed to extend fresh garlic shelf life but it can also reduce characteristic pungent odor and irritating taste and other properties have been changed such as deep brown color and gummy texture together with slightly sweet taste and hints of tamarind. (Figure 6).

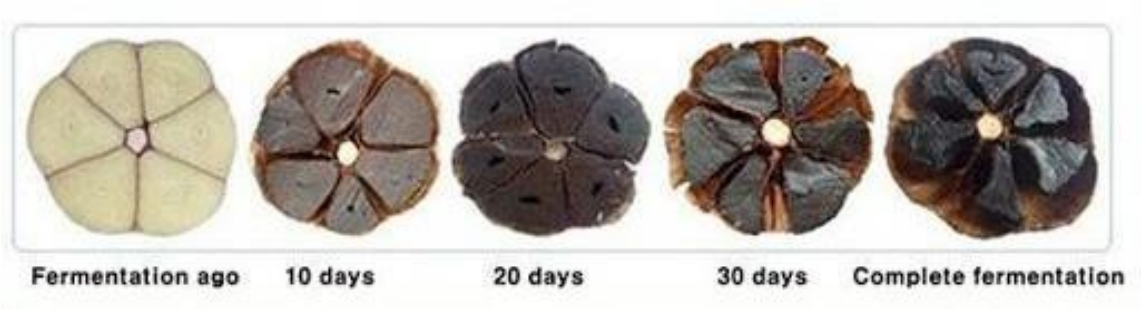


Figure 6 Garlic bulb under thermal treatment at various time intervals

This incubation process causes elevated levels of nutritional amino acids such as leucine, isoleucine, and phenylalanine along with functional metabolites such as polyphenols and flavonoids. In addition, a series of sulfur-containing compounds, which are strong characteristic contributors of flavor and taste of fresh garlic, are also converted; for example, a key pungent odor γ-glutamylcysteine is converted to *S*-allylcysteine [15]. (Figure 7).

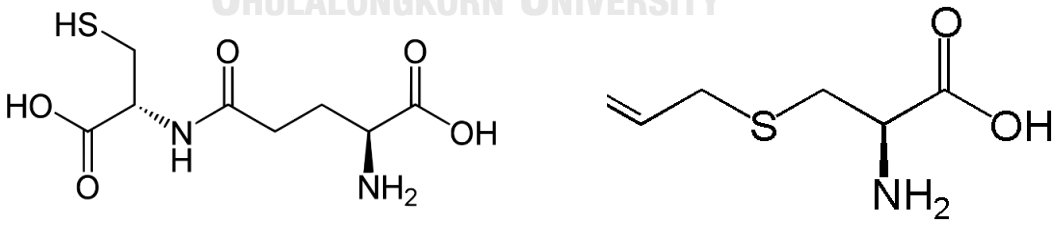


Figure 7 Structures of γ-glutamylcysteine (left) and *S*-allylcysteine (right)

During the black garlic processing, there are several reactions involved, the main chemical reaction is Maillard's reaction [16]. It is a non-enzymatic browning reaction occurs between amine groups of amino acid and carbonyl group of reducing sugars. This is the source of smell and specific properties in black garlic and other heat-treated food such as coffee, steak, bread, roasted cocoa, and pastries. There are several compounds obtained from this reaction such as 5-HMF and melanoidin. It has been reported that both of them has biological effect including antioxidant activity.

In addition to chemical change in BG, several pharmacological and biological activities have also been reported for example anti-inflammatory, anticancer, anti-allergic, antidiabetic, hepatoprotective, cardioprotective, neuroprotective and obesity preventing properties [17]. Moreover, BG is also applicable in cosmetic industry based on its good antioxidant activity such as cream, serum, soap, lotion, conditioner, and shampoo.

1.2.2 Pharmacological activity

Antioxidant

Several studies show that black garlic has high antioxidant content like total polyphenols and total flavonoids. In addition, further studies indicated that BG contains various other antioxidants, such as *N*-fructosyl glutamate, *N*-fructosyl arginine, allicin, and pyruvate. Moreover, The results of antioxidant activity tests in various assays such as TEAC, DPPH, ABTS, and SOD show that black garlic is a good source of antioxidants and is more effective than fresh garlic. Consequently, much of the pharmacological activity of black garlic may come from its antioxidant properties, as in the research of Balamash and coworkers (2012) [18]. They collected blood samples from 48 diabetic patients who consumed 3000 mg of black garlic extract daily over a period of three months. Serum triglycerides, advanced glycation end products (AGEs), and lipid hydroperoxides were analyzed, with higher AGEs being linked to a higher risk of chronic complications. Lipid hydroperoxide is an indicator of oxidative stress. The

results show that the levels of serum triglycerides, advanced glycation end products (AGEs), and lipid hydroperoxides are decreased. As a result, black garlic extract may protect against the negative effects of AGEs and oxidative stress.

Anticancer

Nowadays, aged garlic has increasing evidence for anticancer activity, as in the study of Wang and coworkers (2011) [19]. They performed in vitro and in vivo tests to evaluate the effect of black garlic extract. The results show that in vitro on SGC-7901 (human gastric carcinoma cell) black garlic can induce apoptosis in a dose dependent manner. Additionally, in vivo studies using mice as the model, tumor volume and weight were reduced in the treated group in comparison to the untreated group. In a different investigation, HT29 (colon cancer cell) was used to assess how well black garlic inhibited cell proliferation. The results demonstrate that it can, in a dose-dependent manner, block HT29 cell growth by inducing apoptosis and cell cycle arrest [20]. In 2006, Tanaka and coworkers carried out a clinical trial by oral intake of black garlic extract for 12 months on 51 patients with colorectal adenomas, a precursor lesion of colorectal adenocarcinoma (colon cancer). The number and size of adenomas were counted using colonoscopy and evaluated for the efficacy of black garlic extract. The results showed the suppression of both the size and number of adenomas in the active group. This indicated that the black garlic extract could suppress the progression of colorectal adenomas [21].

Antidiabetes

Traditional medicine claims that black garlic has antidiabetic activity which has been confirmed by current research. Most of the results show that its efficacy is related to antioxidant activity. In 2017, Kim and coworkers [22] evaluated antidiabetic efficacy of black garlic in streptozotocin-induced diabetic mices. Blood sugar level in BG treated mices was reduced significantly compared with untreated group. In addition, BG extract

also showed protective effect over islets of langerhans possibly through antioxidant activity contributed by high polyphenol content. According to Prihanti and colleagues in 2019 [23] and Thomson and colleagues in 2015 [24], they conducted in vivo tests using rats as diabetes models. The results showed that black garlic extract can lower blood glucose levels in rats. In 2009, Seo and coworkers [25] reported that BG is beneficial in the treatment of type II diabetes. BG could increase the sensitivity of insulin in animal models and reduce total serum cholesterol and triglycerides. In 2009, Lee and coworkers [26] evaluated antioxidant activity of BG and raw garlic, both in vitro and in vivo. BG showed high antioxidant activity as evidenced by higher values of trolox equivalent antioxidant capacity (TEAC), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. Therefore, BG can prevent diabetes complications in type 2 diabetic mice by promoting the action of enzymes involved in antioxidants activities. Although various investigations showed that black garlic can decrease blood glucose level, the investigation performed by Balamash and colleagues in 2012 demonstrated controversial result. The blood samples collected from diabetic patients who intake black garlic extract daily for three months showed no change in glucose level. Therefore, additional study is required to clarify this problem and confirm the mechanism of action, usage dose along with active compounds for maximum effectiveness and safety

Prevention of diabetes complications

Cardiovascular disease is a group of diseases that are associated with heart and vascular disorders. People with hypertension, hyperlipidemia, obesity, and hyperglycemia have a higher risk of cardiovascular disease. Previous research indicates that aged garlic can prevent the progress of disease by many mechanisms. In 2019, Hamal and his coworker [27] performed double-blind testing on a patient with type 2 diabetes to investigate the impact of aged garlic extract on endothelial function. An endothelial function disorder portended an increased risk of cardiovascular disease. A total of 65 patients

were tested for the effect of aged garlic extract on endothelial function by the cardio-ankle vascular index (CAVI) technique over a period of three months. The results showed that the aged garlic extract-treated patients had better CAVI values than the placebo group. This finding indicated that AGE has a potential effect on endothelial function and may play a role in the prevention of cardiovascular disease. In another work, they also did an in vivo test in a patient with type 2 diabetes to discover the effect of AGE on reducing low attenuation plaque in coronary arteries. In this study, they used coronary computed tomography angiography (CCTA) for coronary artery pluque volume assessment, and the results showed a statistically significant regression in low attenuation plaque (LAP) when compared to the placebo group [28]. (Shaikh et al., 2019). In the same year, Hutchins and colleagues [29] examined the effect of AGE on reducing left ventricular myocardial mass in patients with diabetes. Increased left ventricular myocardial mass (LVM) is a well-known marker of poor cardiac outcomes. Cardiac computed tomography angiography (CCTA) was used to measure LVM. The results show that the extract group has a significant reduction in LVM and no change was observed in the placebo group.

The consumption of plant-based foods or supplements as a natural source of alpha-glucosidase inhibitors may be more acceptable because they are inexpensive, safe and have fewer gastrointestinal side effects [30]. BG is a plant-based food with a long history of consumption that has been recognized for its safety. Although BG has been reported for lowering blood glucose levels in animal models, identification of active components responsible for such activity along with their inhibitory mechanism has not been documented. Therefore, this study aims to clarify this research gap by applying inhibitory-guided isolation to achieve this goal. The outcome of this project would provide information for consumers to use in the control and prevention of diabetes and its possible complications, as well as for further production of functional food.

1.3 Scope of research

The active fractions of black garlic extract will be fractionationed and purified to afford major active compounds, whose structures will be characterized by spectroscopic methods and the active compounds will be evaluated for in vitro α-glucosidase inhibition together with inhibitory mechanism.

1.4 Expected beneficial outcomes

Active compounds responsible for in vitro α-glucosidase inhibition from black garlic will be obtained.

Chapter 2

Experimental

2.1 Apparatus and reagents

Analytical thin layer chromatography (TLC) was performed on precoated Salicycle siliaplate F_{254} and Merck silica gel 60 F_{254} plates (0.25 mm thick layer) and visualized by UV light (λ = 254 nm) and anisaldehyde reagent. Column chromatography was performed by Merck silica gel 60 (70-230 mesh) and Sephadex LH-20. Evaluation of α-glucosidase inhibitory assay validated by Tecan infinite F50 and performed with rat intestine acetone powder obtained from Sigma-Aldrich (St. Louis, MO, USA), Glucose assay kit was obtained from Human Gesellchaft für Biochemica und Diagnostica mbH (Germany). Acarbose was obtained from Bayer (Germany). Incubator was performed by Allsheng MB100-2A thermos-shaker. ^{1}H NMR spectra were recorded on JEOL JNM-ECZ500R/S1 NMR spectrometers operating at 500 MHz.

2.2 Preparation of black garlic extracts

Black garlic bulbs were purchased from B-garlic (Nopphada Products Co,. Ltd. Lamphun, Thailand) in 2020. They were peeled, ground with silica gel in a ratio of 1:1 and extracted with methanol. The methanol extract was first diluted with deionized water and partitioned with hexane. The hexane layer was concentrated with rotary evaporator to yield hexane extract while the MeOH-H_2O layer was further diluted with water and subsequently partitioned with ethyl acetate. The organic layer was concentrated to afford ethyl acetate extract, and the remaining layer was thermally evaporated (60-70 °C) to yield aqueous extract.

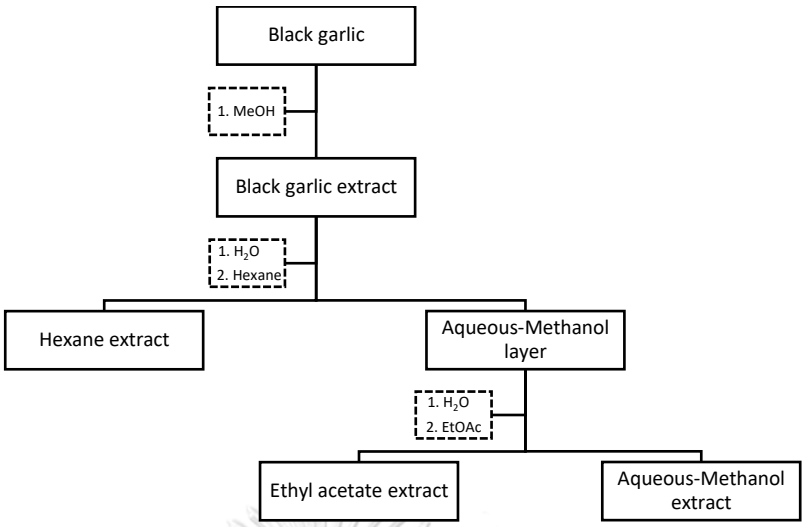


Figure 8 Extraction plan of black garlic

2.3 Isolation, purification, and structure elucidation

The extracts active against α-glucosidase activity will be further fractioned isolated and purified mainly by suitable chromatography followed by Bioassay-guided isolation using bioassay as a guide. The structures of isolated pure compounds will be characterized by spectroscopy techniques such as NMR or authenticated by TLC comparison with authentic samples.

2.4 Preparation of α-glucosidase

Rat intestinal acetone powder from Sigma-Aldrich (St. Louis, MO, USA) was mixed with 9 %w/v normal saline in a ratio of 1 g. per 30 ml. Then, the centrifugation of the mixture was performed at 12,000 rpm for 30 minutes. The liquid part was obtained as the enzyme solution.

2.5 Rat intestinal of α-glucosidase inhibitory assay

α-Glucosidase inhibition of isolated compounds was evaluated using colorimetric method previously described by Worawalai et al. [31]. Principally, sucrose is

catalytically hydrolyzed by sucrase to yield two monosaccharides glucose and fructose. Glucose is subsequently oxidized by glucose oxidase to produce gluconic acid and H_2O_2; the latter of which continuously oxidized 4-aminophenazone (colorless) in the presence of peroxidase to afford quinoneimine (purple) (Figure 9). The sucrase activity can be quantified by colorimetry at the wavelength of 520 nm. The maltase activity can be also evaluated using the same protocol applied for sucrase activity.

The enzyme solution was as a source of sucrase and maltase. It (20 μl) were added with phosphate buffer (pH 6.9, 30 μl), substrate solution (maltose 10 mM / sucrose 100 mM, 20 μl), Isolated compound (10 μl), and glucose assay kit (SU-GLLQ2, Human, 80 μl). After that, the mixture was incubated at 37°C for 10 minute (maltose) and 40 minute (sucrose). Acarbose were used as positive control and the assay performed in triplicate. The absorbance of quinoneimine was measured at 520 nm using Tecan infinite F50 and the percentage of inhibition was calculated by mean of [(A0-A1)/A0] * 100 (A0: absorbance without sample and A1: absorbance with sample). Percentage inhibition and sample concentration were used to estimate the IC_{50} value.

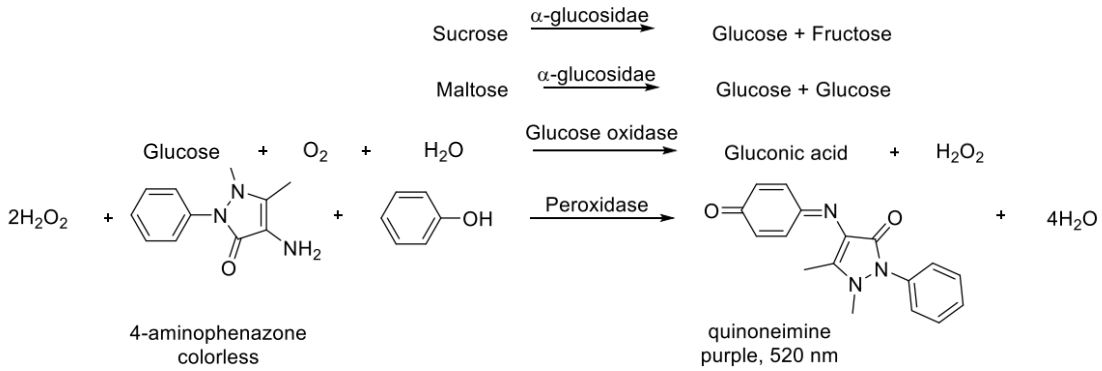


Figure 9 Colorimetric technique to quantify α-glucosidase inhibition

2.6 Kinetic study of α-glucosidase inhibition

To provide that which mechanism responsible for each isolated active compound, kinetic study was performed by colorimetric method previously described by Worawalai et al. by varying substrate concentration both maltose and sucrose in the absence and presence of glucose. The enzyme solution was used as a source of sucrase and maltase. It (20 μl) were added with phosphate buffer (pH 6.9, 30 μl), increased substrate solution (maltose 2-10 mM / sucrose 20-100 mM, 20 μl), Isolated compound (10 μl), and glucose assay kit (SU-GLLQ2, Human, 80 μl). After that, the mixture was incubated at 37ºC for 10 minute (maltose) and 40 minute (sucrose). Acarbose were used as positive control and the assay performed in triplicate. The absorbance of quinoneimine was measured at 520 nm using Tecan infinite F50. The absorbance data from microplate reader used to analyze mechanism of inhibition of active compound that were calculated by Lineweaver Burk linearization.

The Lineweaver Burk plot equation in double reciprocal form can be written as:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

Secondary plots can be constructed from:

$$Slope = \frac{K_m}{V_{max}} + \frac{K_m[I]}{V_{max}K_i}$$

$$Y - intercept = \frac{1}{V_{max}} + \frac{[I]}{\alpha K'_i V_{max}}$$

The kinetic parameter

V_0: initial velocity of an enzyme reaction

V_{max}: the maximum of an enzyme catalyzed reaction

K_m: Michaelis constant

K_i: The value of the dissociation constant for the enzyme-inhibitor complex

K'_i: The value of the dissociation constant for the enzyme-substrate-inhibitor complex

S: Substrate concentration

I: Inhibitor concentration

Chapter 3

Results and Discussion

3.1 α-Glucosidase inhibition screening of crude extracts

From the extraction scheme of black garlic shown in Figure 10, three extracts were obtained. They were hexane (21.2 g), ethyl acetate (43.5 g), and aqueous methanol (1682.2 g) extracts. Selecting the active extract for further study was done using bioassay-guided isolation. The results of α-glucosidase inhibition are shown in Figure 11. The higher the efficiency against α-glucosidase, the lower IC_{50} value. Of the extracts obtained, ethyl acetate and aqueous-methanol extracts showed dose-dependent inhibition against maltase and sucrase with IC_{50} values in ranges of 438.25-1415 mg/mL. For hexane extract, it showed weaker inhibition against sucrase with IC_{50} value of -572.24 and 3163.93 mg/mL. On the other hand, a negative inhibition against maltase possibly resulted from oil characteristic of hexane extract that made unclear solution during absorbance measurement. Based on the screening results, the ethyl acetate and aqueous-methanol extracts from the black garlic were further fractioned and purified.

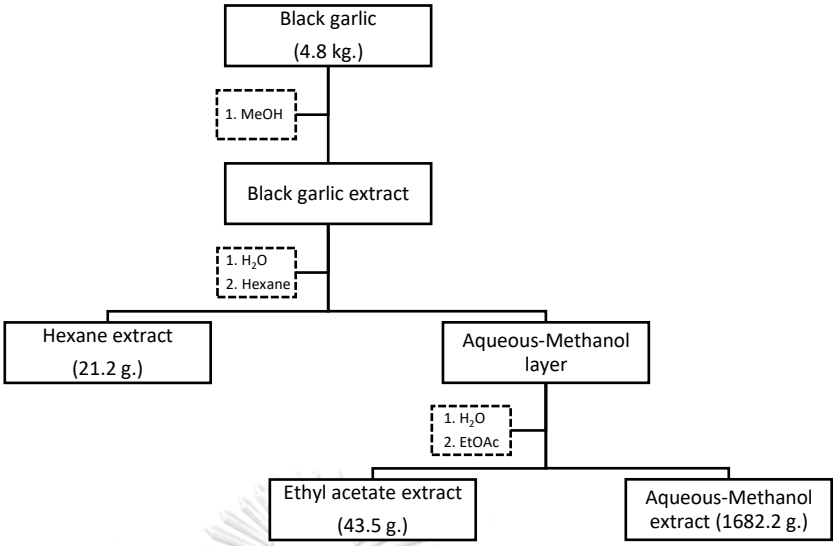
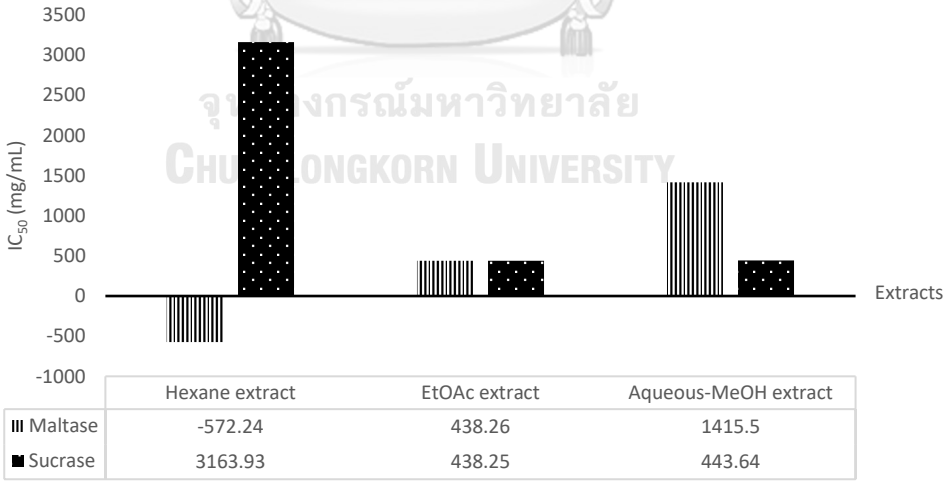


Figure 10 Extraction scheme of black garlic



	Hexane extract	EtOAc extract	Aqueous-MeOH extract
Maltase	-572.24	438.26	1415.5
Sucrase	3163.93	438.25	443.64

Figure 11 **α**-Glucosidase inhibition of the extracts expressing in terms of IC_{50} values

3.2 Bioassay-guided isolation of ethyl acetate extract

3.2.1 Fractionation

The ethyl acetate extract (43.5 g.) was separated by silica gel column chromatography using the following mobile phases namely mixtures of ethyl acetate-hexane (50:50 and 75:25) and methanol-ethyl acetate (5:95 and 20:80). According to TLC profiles, six combined fractions (**E1**-**E6**) were obtained.

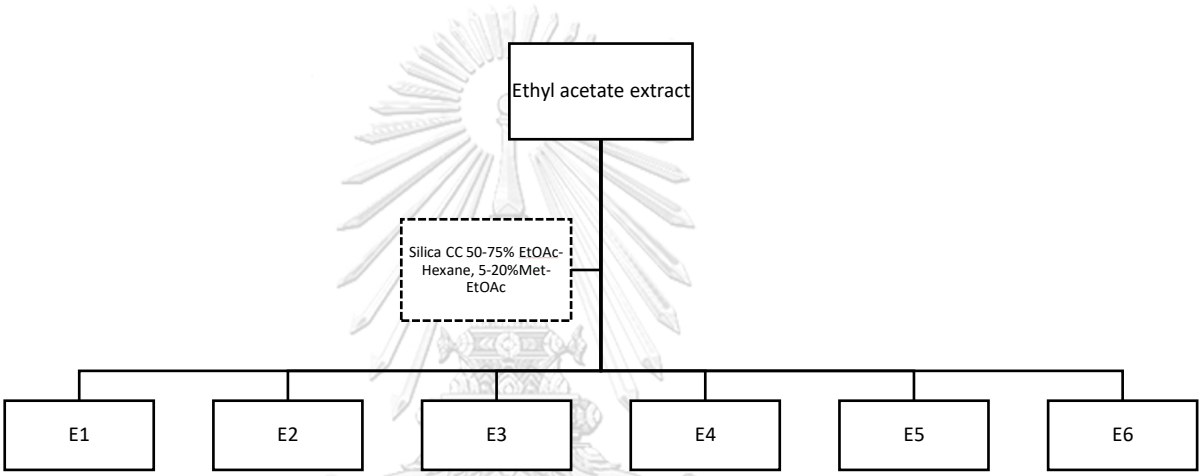
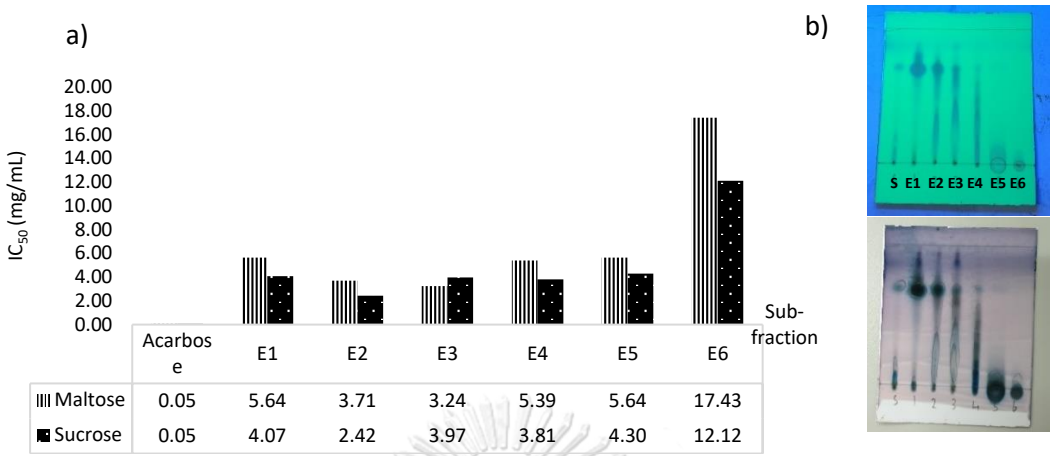


Figure 12 Separation of ethyl acetate extract

3.2.2 **α**-Glucosidase inhibitory evaluation of fractions **E1**-**E6**

The fractions **E1**-**E6** obtained after fractionation of ethyl acetate extract were evaluated for **α**-glucosidase inhibition, and the results are shown in Figure 13. Obviously, fractions **E1**-**E5** demonstrated 2-3 time stronger inhibition (IC_{50} 2.42-5.64 mg/mL) than that of fraction **E6** (IC_{50} 12.12-17.43 mg/mL). Of fractions **E1**-**E5**, fraction **E2** showed highest inhibition, and the TLC profile demonstrated the presence of single major spot. In addition, fraction **E5** also showed strong inhibition and its TLC profile was quite different from those of **E1**-**E4**. Therefore, fractions **E2** and **E5** were further purified to get the pure compounds.



	Acarbose	E1	E2	E3	E4	E5	E6
Maltose	0.05	5.64	3.71	3.24	5.39	5.64	17.43
Sucrose	0.05	4.07	2.42	3.97	3.81	4.30	12.12

Figure 13 a) **α**-Glucosidase inhibition and b) TLC profile of fractions **E1**-**E6**

3.2.3 Isolation and purification of fractions **E2** and **E5**

Fraction **E2** was purified by silica gel column chromatography eluted with ethyl acetate-hexane (1:3 v/v) to obtain seven subfractions (**E2.1**-**E2.7**, Figure 14). The pure compound was obtained in fraction **E2.5**. Fraction **E5** was repeatedly separated by Sephadex LH-20 column using methanol as a mobile phase to afford 4 subfractions (**E5.1**-**E5.4**, Figure 15). Subfraction **E5.3** was further purified using silica gel column chromatography eluted with methanol-ethyl acetate (5:95) to afford single pure compound in subfraction **E5.3.3**.

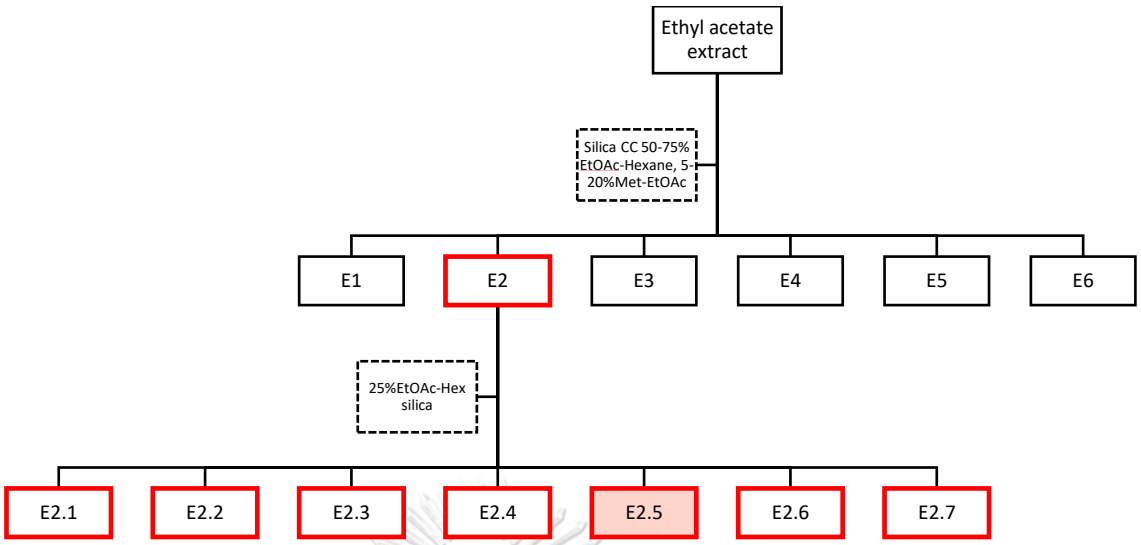


Figure 14 Separation of fraction **E2**

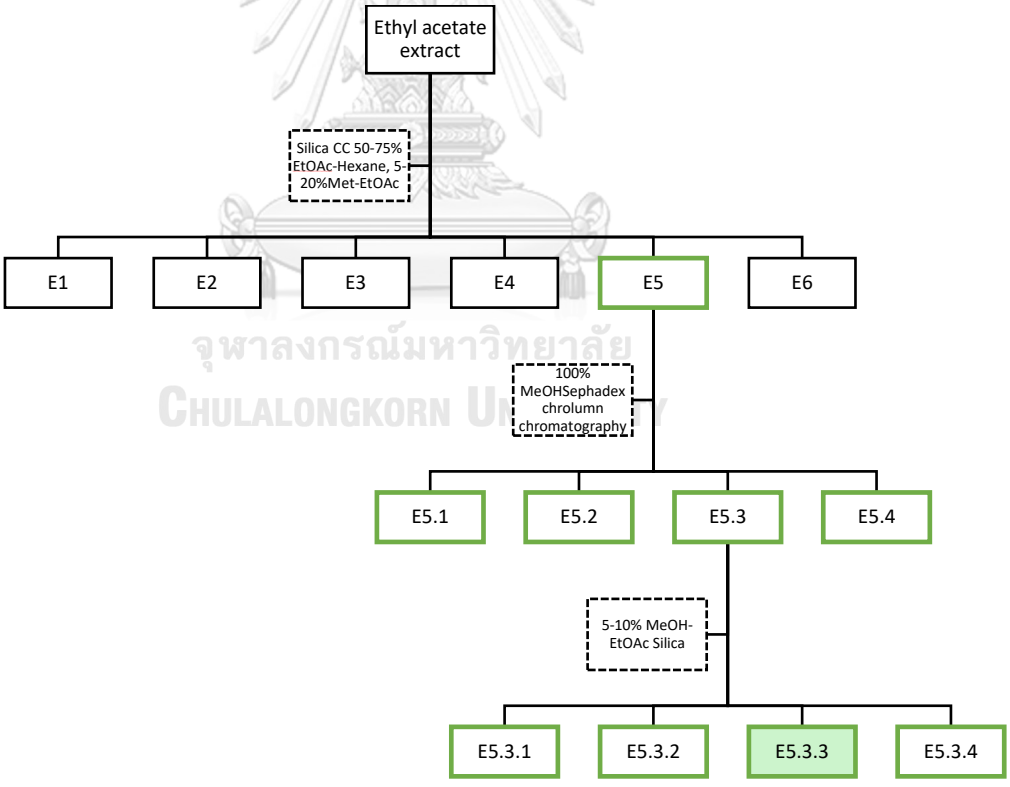


Figure 15 Separation of fraction **E5**

3.2.4 Structure elucidation of **E2.5**

Compound **E2.5** was obtained as dark brown sticky liquid. It showed the singlet signal of aldehyde (-CHO) at 9.53 (s), a furan moiety at 7.20 (d, *J* = 3.3 Hz) and 6.49 (d, *J* = 3.1 Hz) and oxygenated methylene at 4.74 (s) (Figure 3.7). The ^{1}H NMR data clearly indicated the identity of 5-hydroxymethyl furfural (5-HMF) (Figure 3.8) and coincided well with those reported by Campo and coworker (2020) [32].

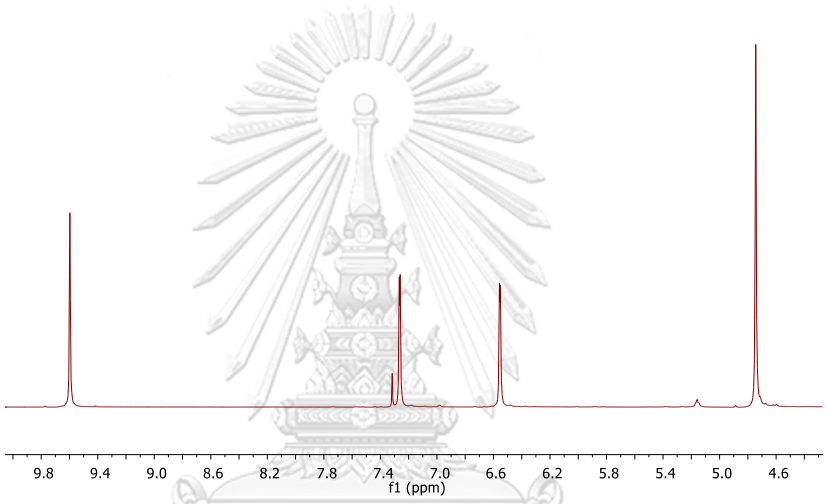


Figure 16 ^{1}H NMR spectrum of **E2.5** in $CDCl_3$ (500 MHz)

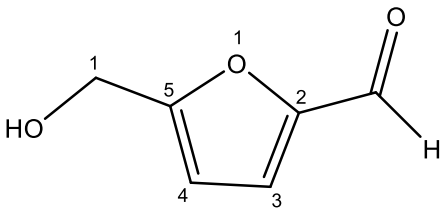


Figure 17 Chemical structure of 5-hydroxymethyl furfural

3.2.5 Structure elucidation of **E5.3.3**

Compound **E5.3.3** was obtained as brown sticky liquid. It showed ^{1}H NMR signals of furan moiety at 7.07 (d, *J* = 3.2 Hz), 6.41 (d, *J* = 3.2 Hz) and oxygenated methylene at 4.53 (s). The ^{1}H NMR of **E5.3.3** was similar to those of **E2.5**, except for the lack of aldehyde signal. The more polar behavior of **E5.3.3** on TLC profile suggested that the aldehyde group in **E2.5** was possibly replaced by carboxylic acid group (-COOH) in **E5.3.3.** This postulation was further verified by ^{13}C NMR data. The presence of quarternary carbon at 160.22 ppm supported the presence of carboxylic acid. Therefore, compound **E5.3.3** was verified as 5-hydroxymethyl-2-furan carboxylic acid or abbreviated as HMFCA (Sayed et al, 2018) [33].

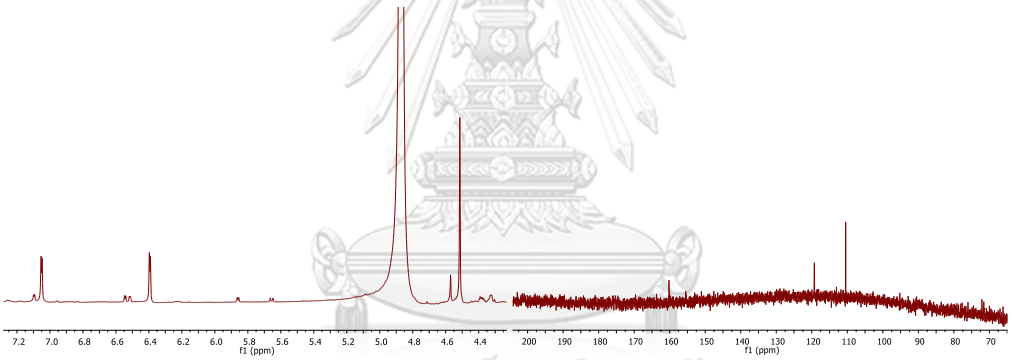


Figure 18 ^{1}H NMR (left) and ^{13}C NMR (right) spectra of **E5.3.3** in methanol-d

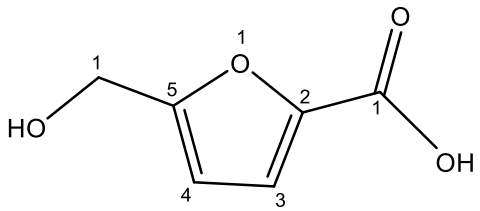


Figure 19 Chemical structure of 5-hydroxymethyl-2-furan carboxylic acid

3.3 Bioassay-guided isolation of aqueous-methanol

3.3.1 Fractionation

The remaining aqueous methanol extract (1682.2 g.) was isolated by silica gel column chromatography eluted with the mixture of methanol and dichloromethane (10:90 to 50:50) to afford six combined fractions (**M1**-**M6**). The isolation procedure is summarized in Figure 20.

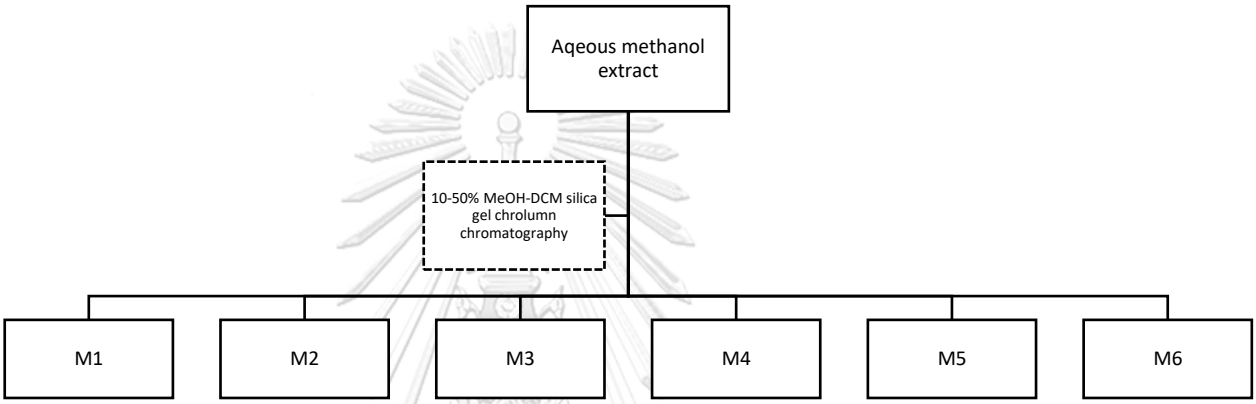


Figure 20 Separation of aqueous-methanol extract

3.3.2 **α**-Glucosidase inhibitory evaluation of fractions **M1**-**M6**

Fractions **M1**-**M6** obtained from aqueous-methanol extract were evaluated for **α**-glucosidase inhibition. The results (Figure 21) are expressed as percentage of inhibition at 2 concentrations, 0.1 and 0.5 mg./mL. The inhibition against sucrase of **M1**-**M6** demonstrated relatively weak inhibition and there was no significant difference among them. On the other hand, the higher inhibition against maltase was clearly observed in fraction **M3** (16.5-17.6%) followed by fraction **M2** (9.4-12.0%). Consequently, fractions **M2** and **M3** were selected for further study.

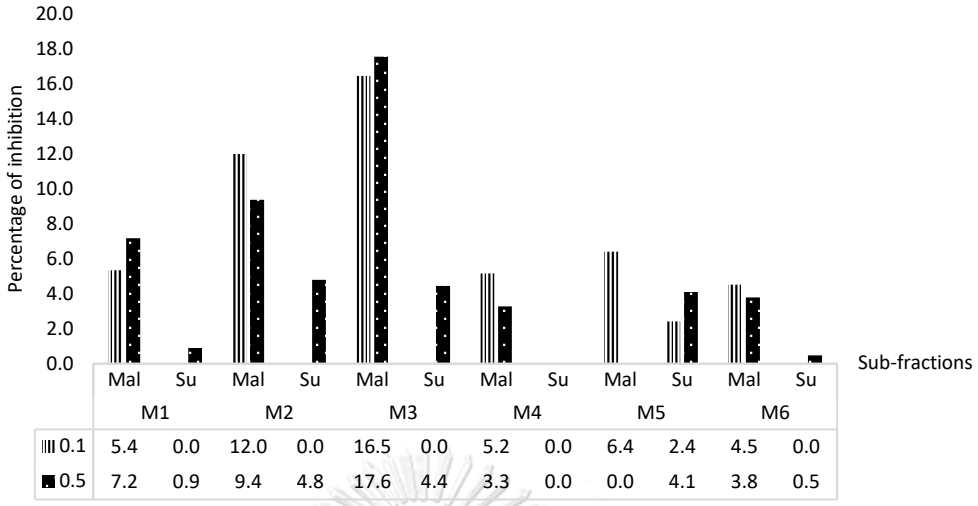


Figure 21 α-Glucosidase inhibition of fractions **M1**-**M6** against maltase and sucrase

3.3.3 Isolation and purification of fractions **M2** and **M3**

From the results of bioactivity in the previous part, fractions **M2** and **M3** were selected for further isolation. Fraction **M2** was fractioned by silica gel column chromatography eluted with methanol-dichloromethane in gradient manner (1:99 to 5:95) to obtain 8 subfractions (**M2.1-M2.8,** Figure 22). Then pure compounds were obtained from subfractions **M2.5** and **M2.8**. Fraction **M3** was applied on silica gel column chromatography eluted with 100% ethyl acetate and mixtures of methanol-ethyl acetate in gradient manner (1:99 to 5:95) to give 4 subfractions (**M3.1-M3.9,** Figure 23). The pure compound was obtained in subfraction **M3.7**.

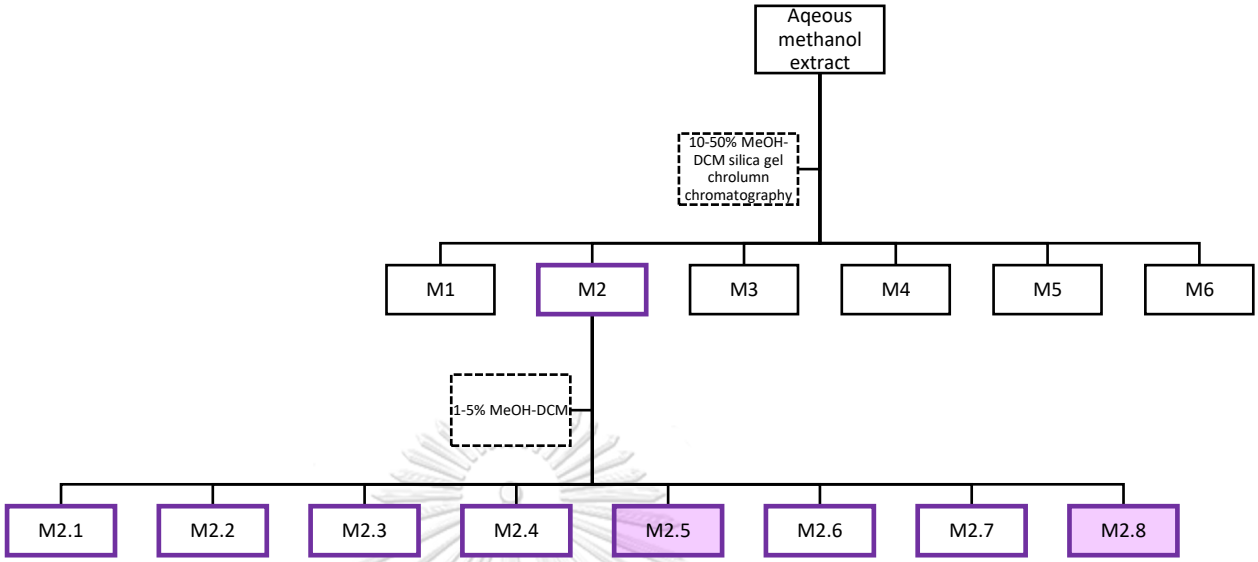


Figure 22 Separation of fraction **M2**

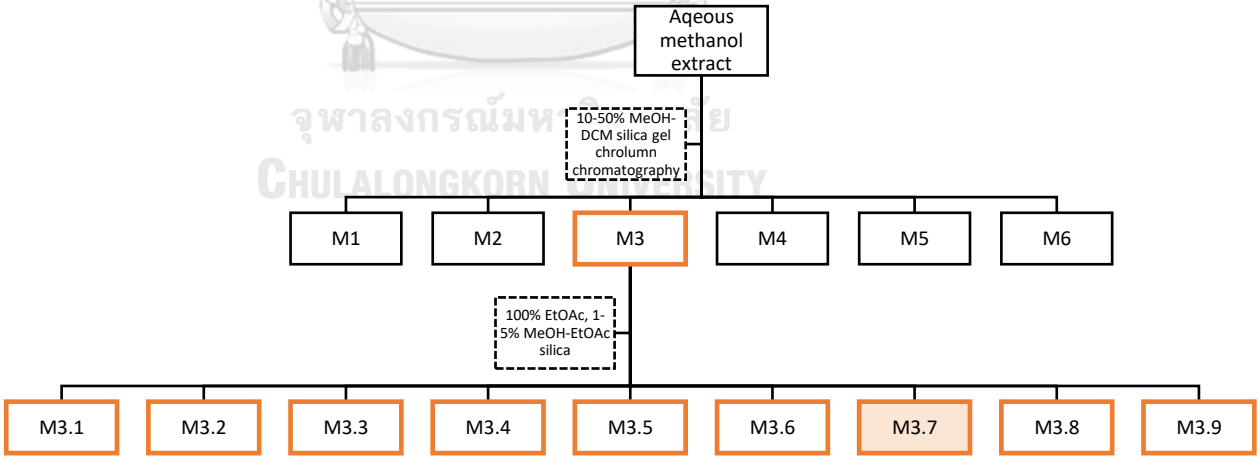


Figure 23 Separation of fraction **M3**

3.3.4 Structure elucidation of **M2.5**

Compound **M2.5** was obtained as colorless substance. It showed ^{1}H NMR signals (Figure 24) of two doublets at 7.17 (d, *J* = 8.6 Hz) and 6.75 (d, *J* = 8.6 Hz), together with a broad signal of hydroxyl group. The characteristic splitting of aromatic signal indicated the presence of disubstituted benzene, whose substitution groups were not identical. The structure of **M2.5** was initially proposed as 4-(hydroxymethyl)phenol, a common natural phenolic found in all kind of plants. Compared with the ^{1}H NMR data of 4-(hydroxymethyl)phenol (Figure 3.16) reported by Yang and coworker (2007) [34], the data of compound **M2.5** were identical. Thus, **M2.5** was verified as 4-(hydroxymethyl)phenol.

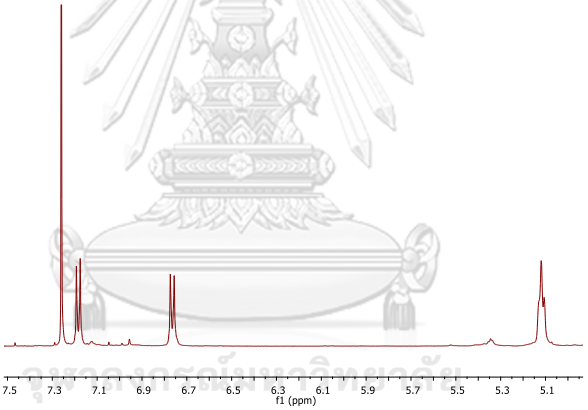


Figure 24 ^{1}H NMR spectrum of **M2.5** in chloroform-d

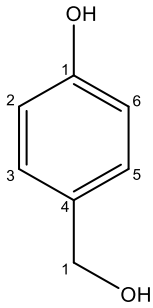


Figure 25 Chemical structure of 4-(hydroxymethyl)phenol

3.3.5 Structure elucidation of **M2.8**

Compound **M2.8** was obtained as dark brown color substance It show ^{1}H NMR signals at δ (ppm) 4.70 (s), 6.50 (d, *J* = 3.5 Hz), 7.21 (d, *J* = 3.5 Hz), and 9.58 (s). The NMR data of **M2.8** was nearly identical to that of **E2.5** while direct comparison of **M2.8** and **E2.5** on TLC revealed the same spot. Therefore, **M2.8** was identified as 5-hydroxymethyl furfural (5-HMF).

3.3.6 Structure elucidation of **M3.7**

Compound **M3.7** was obtained as brown color substance. It showed ^{1}H NMR signals (Figure 26) at δ (ppm) 4.40 (dt, 6.7, 6.7 Hz), 3.73 (ddd, *J* = 3.3, 3.3, 3.6, 3.6), 2.92 (dd, *J* = 6.7, 6.7 Hz), 2.33 (dd, *J* = 2.5, 2.5 Hz). These data preliminarily suggested that this compound contained several oxygenated methane (-OCH-) and methylene (-OCH_2-). Compared to the NMR data reported by Voituriez and Cadet (1999) [35] [36], **M3.7** was identified as 2-deoxy-ribono-1,4 lactone (Figure 27).

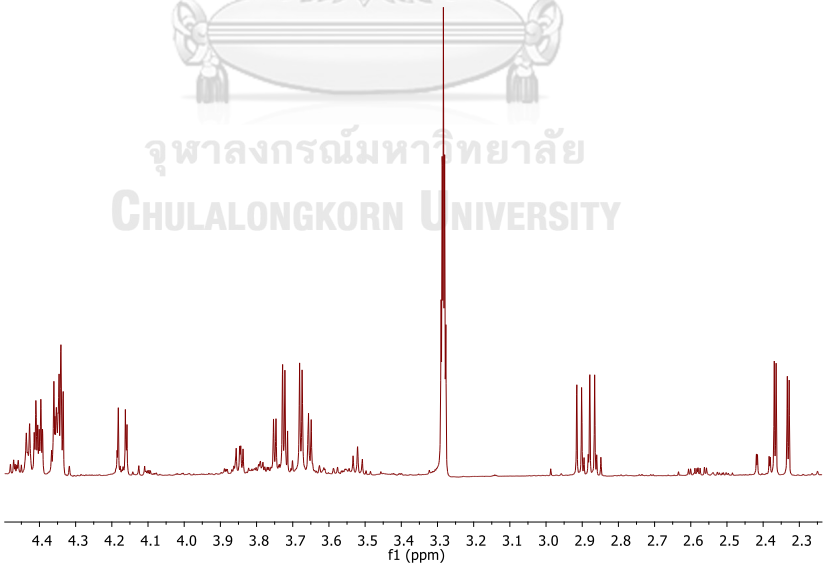


Figure 26 ^{1}H NMR spectrum of **M3.7** in chloroform-d

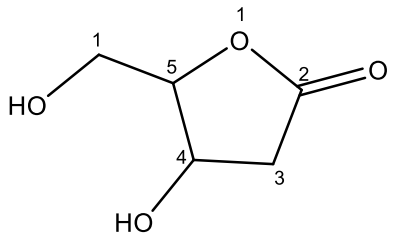


Figure 27 Chemical structure of 2-deoxy-ribono-1,4 lactone

In this experiment, the inhibitory activity against **α**-glucosidase was used as a guide for selecting the active extracts and fractions for further isolation. Totally, four pure substances were isolated from the crude extracts, consisting of 5-hydroxymethyl furfural, 5-hydroxymethyl-2-furan carboxylic acid, 4-(hydroxymethyl)phenol, and 2-deoxy-ribono-1,4 lactone. The overview separation diagram is depicted in Figure 28.

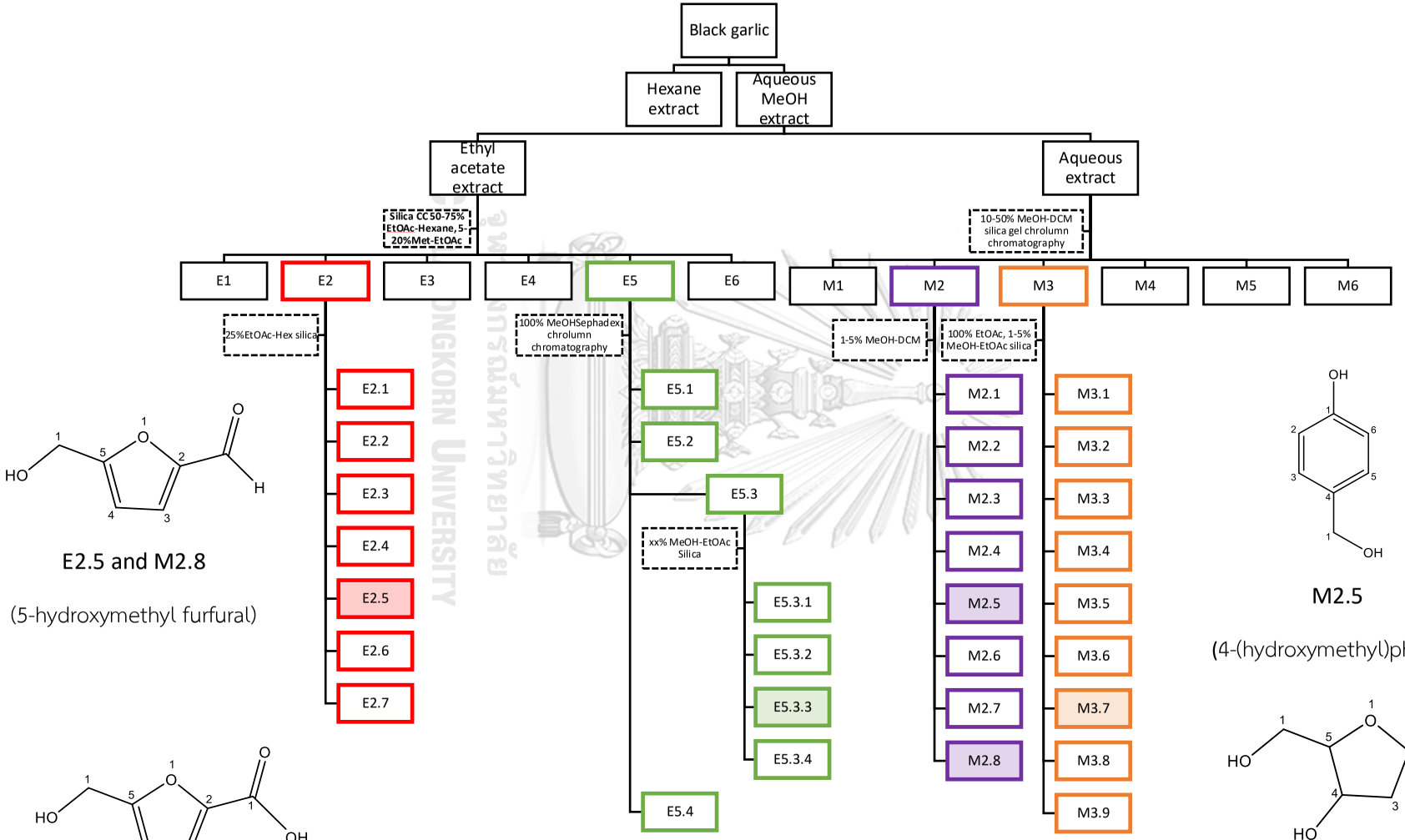


Figure 28 separation diagram

E2.5 and M2.8

(5-hydroxymethyl furfural)

E5.3.3

(5-hydroxymethyl-2-furan carboxylic acid)

M2.5

(4-(hydroxymethyl)phenol)

M3.7

(2-deoxy-ribono-1,4 lactone)

3.4 The chemical relation among isolated metabolites

Black garlic is a health product derived from the process of aging fresh garlic under controlled conditions of high temperature and humidity to achieve the desired product characteristics. It is dark brown, sweet taste, and has a reduced pungent odor. Due to this production process, the constituents present in fresh garlic may decompose due to high heat. This study obtained totally 4 compounds including 5-HMF (**E2.5** and **M2.8**), HMFCA (**E5.3.3**), 4-(hydroxymethyl)phenol (**M2.5**) and 2-deoxy-ribono-1,4 lactone (**M3.7**). They are small molecule and are not commonly found in fresh garlic. Therefore, these compounds may be obtained by decomposing other substances in fresh garlic during the black garlic processing. From the experiment, some compounds may be the product of carbohydrate degradation during the thermal process. As previously researched, 5-HMF can be transformed from c6 sugar like fructose or glucose, which are the main components of black garlic [37]. From the research of Lalanne and coworkers (2021) [38], HMFCA is a compound in the same group as 5-HMF and it can be obtained from 5-HMF by oxidation reaction. For 4-(hydroxymethyl)phenol, it has the possibility to be transformed from tyrosine, an amino acid found mainly in fresh garlic and lower in black garlic [39]. In addition, 5-HMF was found from both ethyl acetate and aqueous-methanol extracts, indicating that it is one of the major components in black garlic. It has a dark brown color that is responsible for the characteristics of black garlic. 5-HMF is a substance that is formed by the Maillard reaction during the heating process in the production of many types of food and beverages that are grilled, roasted, and baked. It is a reaction between amino acids and reducing sugar. 5-HMF is a substance that changes certain characteristics of food, such as its dark color and different nutritional values [40]. During the black garlic production process, it was found that the more time it took to incubate, the higher the 5-HMF content [41]. According to Zhang and coworkers (2016) [42], the amount of this substance may be used as one of the indicators in black garlic

production. They used 5-HMF to indicate the maturity of black garlic during the production process.

3.5 Rat intestine α-glucosidase inhibition of isolated compounds

All isolated compounds were evaluated for α-glucosidase inhibition against rat intestinal maltase and sucrase (Table 1). In this experiment, 5-HMF showed no inhibition (IC_{50} more than 30 mM) in both maltase and sucrase, while HMFCA demonstrated inhibitory effect against maltase and sucrase with IC_{50} values of 18.83±0.91 and 17.42±0.84 mM, respectively. One point of interest in this experiment was the slightly altered structure of 5-HMF to HMFCA, conversion of functional groups from aldehyde to carboxylic. It was found that the inhibitory activity against α-glucosidase was significantly changed. It was indicative that carboxylic acid plays an important role in inhibiting α-glucosidase function, and this is the first report of inhibitory effect of HMFCA against these enzymes. Modification of carboxylic acid moiety would lead to the discovery of a new series of potent α-glucosidase inhibitors. Moreover, 4-(hydroxymethyl) phenol also inhibited maltase and sucrase with IC_{50} values of 23.70±1.52 and 6.78±0.17 mM, respectively. However, 2-deoxy-ribono-1,4-lactone has an inhibitory effect against maltase only with IC_{50} value of 9.26±0.31 mM, whereas it was not active against sucrase.

Table 1 α-Glucosidase inhibition of isolated compounds

Compounds	IC_{50}	
	Maltase (mM)	Sucrase (mM)
5-hydroxymethyl furfural	NI[a]	NI[a]
5-hydroxymethyl-2-furan carboxylic acid	18.83±0.91	17.42±0.84
4(hydroxymethyl)phenol	23.70±1.52	6.78±0.17
2-deoxy-ribono-1,4 lactone	9.26±0.31	NI[a]
Acarbose[b]	0.85±0.17	1.07±0.18

[a]NI are $IC_{50} \geq 30$ or dose independent, [b]Standard control

3.6 Kinetic study of the active compounds 5-hydroxymethyl-2-furan carboxylic acid, 4-(hydroxymethyl)phenol and 2-deoxy-ribono-1,4 lactone

To understand how the isolated compounds inhibit the enzymes, kinetic study was carried out. The Lineweaver-Burk plot was constructed and kinetic parameters were analyzed by varying concentrations of substrates (maltose and sucrose) and isolated compounds. In this study, three active inhibitors namely 5-hydroxymethyl-2-furan carboxylic acid, 4-(hydroxymethyl)phenol and 2-deoxy-ribono-1,4 lactone were investigated.

The Lineweaver-Burk plot of HMFCA (Figure 29.) against maltase displayed a series of straight lines. The parallel of all the straight lines were in the second quadrant. Kinetic examination showed that V_{max} decreased with decreased K_m in the presence of increasing concentrations of HMFCA, supporting that the compound inhibits α-glucosidase in an uncompetitive manner (K_i' of 0.328 mM). The mechanism begins by forming enzyme–substrate (ES) complexes, then substrate–enzyme-inhibitor (ESI) complexes. For the inhibitory mechanism against sucrase was also examined using the above methodology. Apparently, it inhibited these α-glucosidases by uncompetitive manners (K_i' of 7.75 mM). Figure 30.

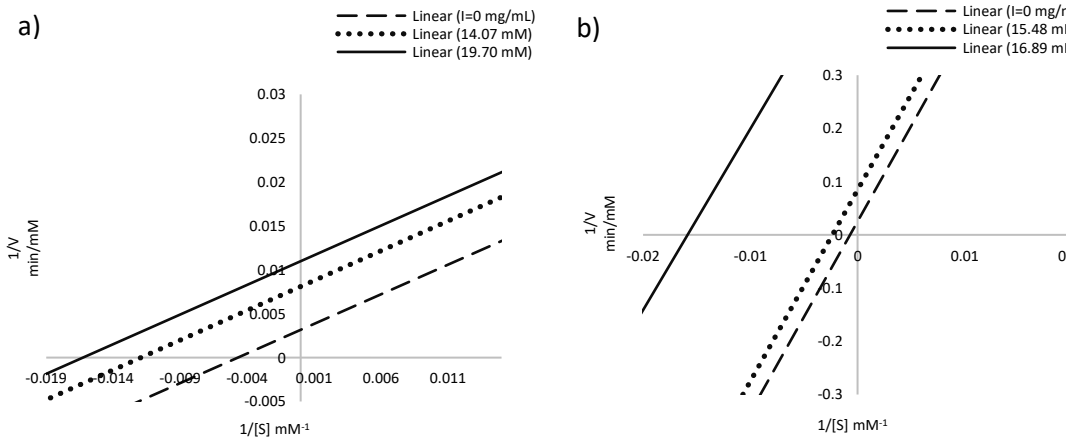


Figure 29 Lineweaver-Burk plots for inhibitory activity of 5-hydroxymethyl-2-furan carboxylic acid against (a) maltase (b) sucrase

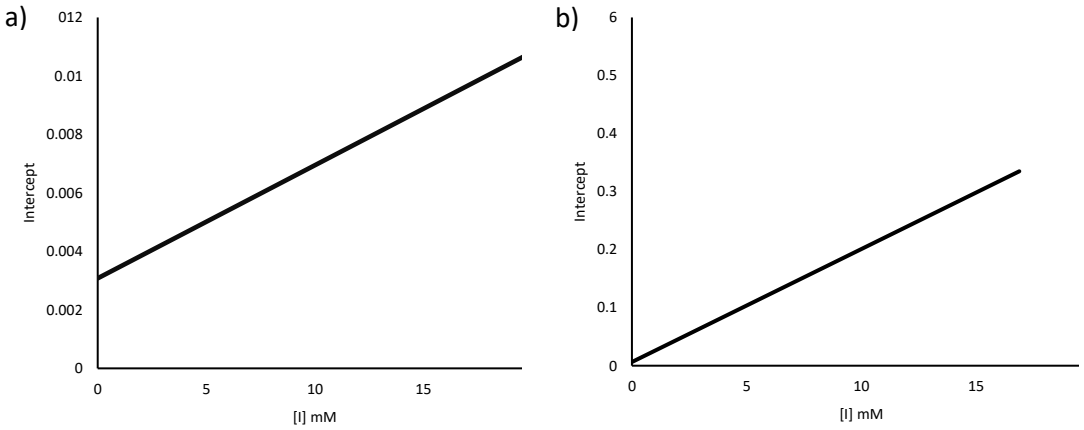


Figure 30 Secondary replot of slope and [I] from a primary Lineweaver-Burk plot for the determination of K_i, of 5-hydroxymethyl-2-furan carboxylic acid

In addition, the kinetic study of 4(hydroxymethyl)phenol for maltase and sucrase (Figure 31) revealed a linear relationship at each tested concentration of it, all of which showed a parallel pattern. The analysis demonstrated that V_{max} and K_m decreased, supporting that it inhibits **α**-glucosidase in an uncompetitive manner in both maltase (K_i' of 7.78 mM) and sucrase (K_i' of 24.89 mM) therefore indicting that the compound predominantly formed a substrate–enzyme-inhibitor (ESI) complex rather than directly bound to enzyme (EI).

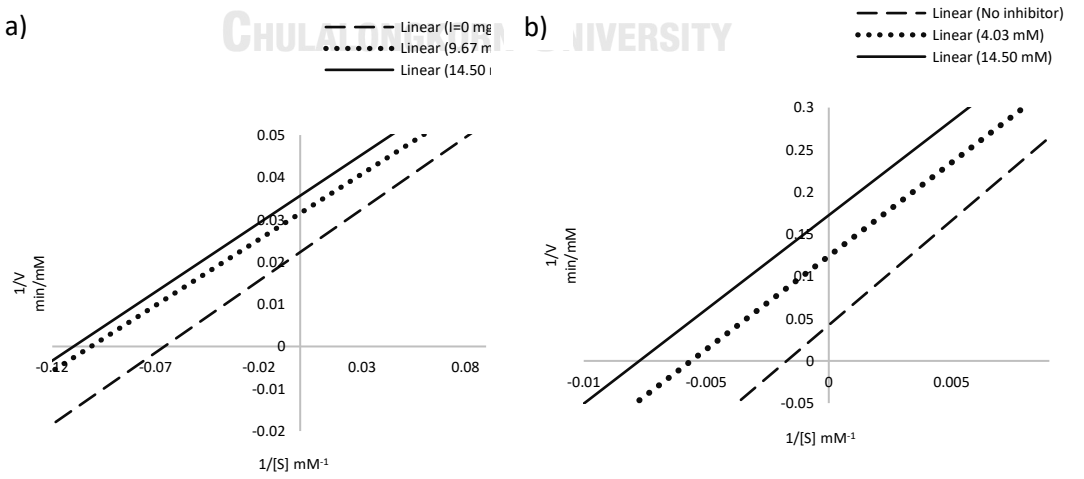


Figure 31 Lineweaver-Burk plots for inhibitory activity of 4(hydroxymethyl)phenol against (a) maltase (b) sucrase

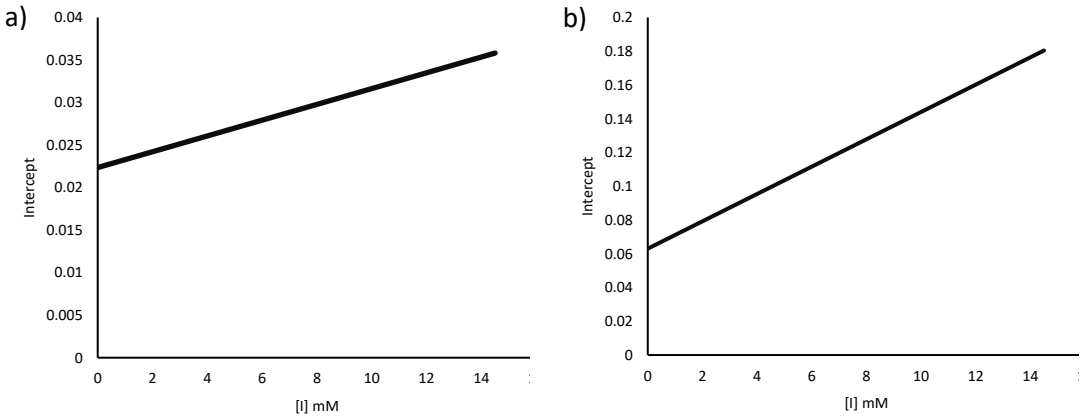


Figure 32 Secondary replot of slope and [I] from a primary Lineweaver-Burk plot for the determination of K_i, of 4(hydroxymethyl)phenol

The Lineweaver–Burk plot of 2-deoxy-ribono-1,4 lactone against maltase (Figure 33) showed a series of straight lines; all of which intersected at the y-axis. Kinetic analysis showed that V_{max} unchange with elevated K_m in the presence of increasing concentrations of the compound. This behavior suggested that maltase could be inhibited by 2-deoxy-ribono-1,4 lactone in competitive manners. The observed result could be elaborated by the simultaneous formation of an enzyme-inhibitor (EI) with the K_i value of 37.96 mM.

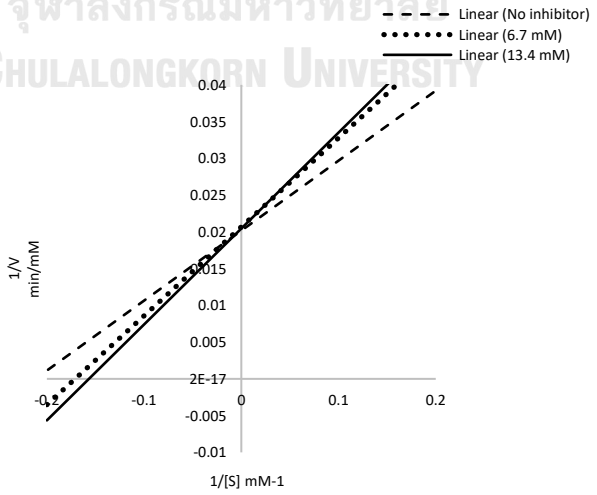


Figure 33 Lineweaver-Burk plots for inhibitory activity of 2-deoxy-ribono-1,4 lactone against maltase

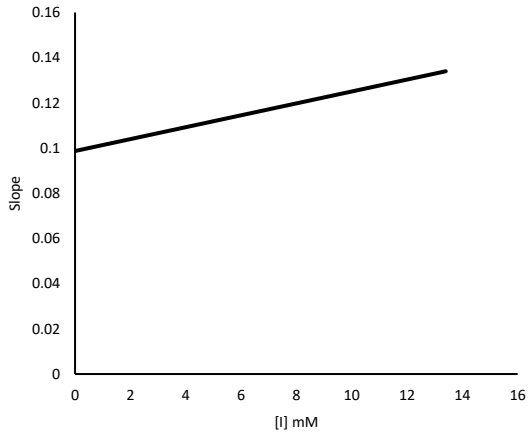


Figure 34 Secondary replot of slope and [I] from a primary Lineweaver-Burk plot for the determination of K_i' of 2-deoxy-ribono-1,4 lactone

Table 2 Inhibition type and kinetic parameters of 5-Hydroxymethyl-2-furan carboxylic acid, 4(hydroxymethyl)phenol, and 2-deoxy-ribono-1,4 lactone

Compounds		Maltase	Sucrase
5-hydroxymethyl-2-furan carboxylic acid	Inhibition type	Uncompetitive	Uncompetitive
	K_i	-	-
	K_i'	0.33	7.75
4(hydroxymethyl)phenol	Inhibition type	Uncompetitive	Uncompetitive
	K_i	-	-
	K_i'	7.78	24.89
2-deoxy-ribono-1,4 lactone	Inhibition type	competitive	-
	K_i	37.96	-
	K_i'	-	-

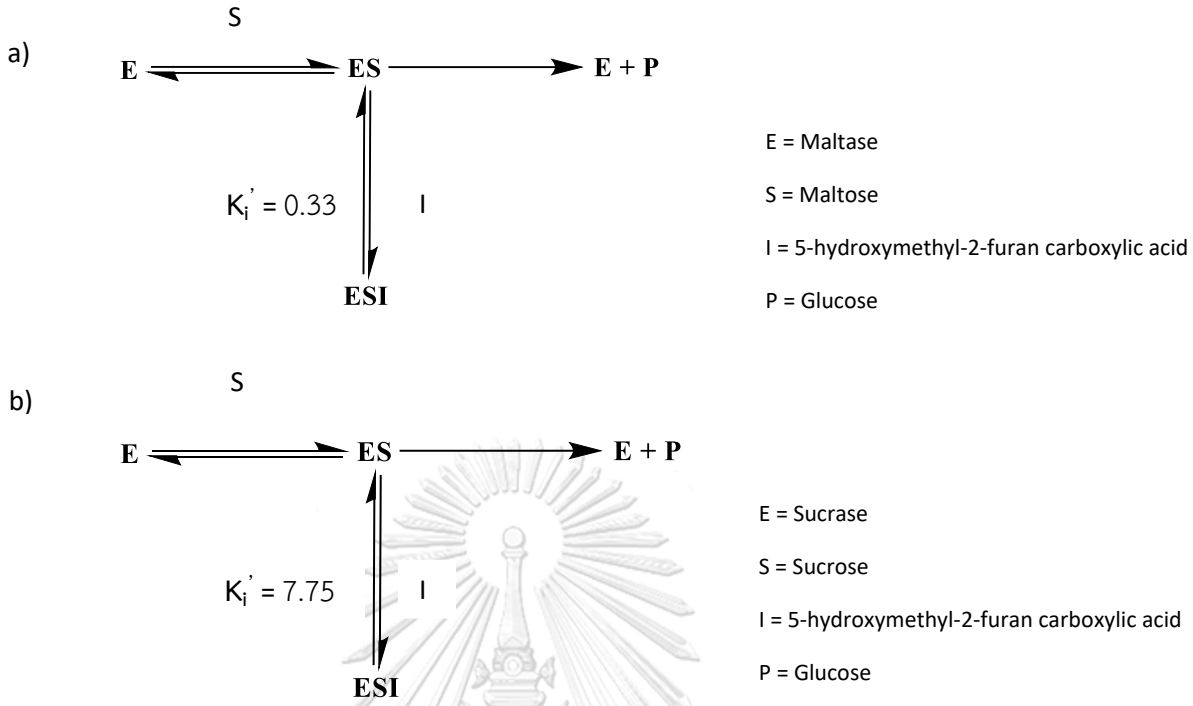


Figure 35 Putative mechanism pathway of 5-hydroxymethyl-2-furan carboxylic acid for uncompetitive inhibition against maltase(a) and sucrase(b)

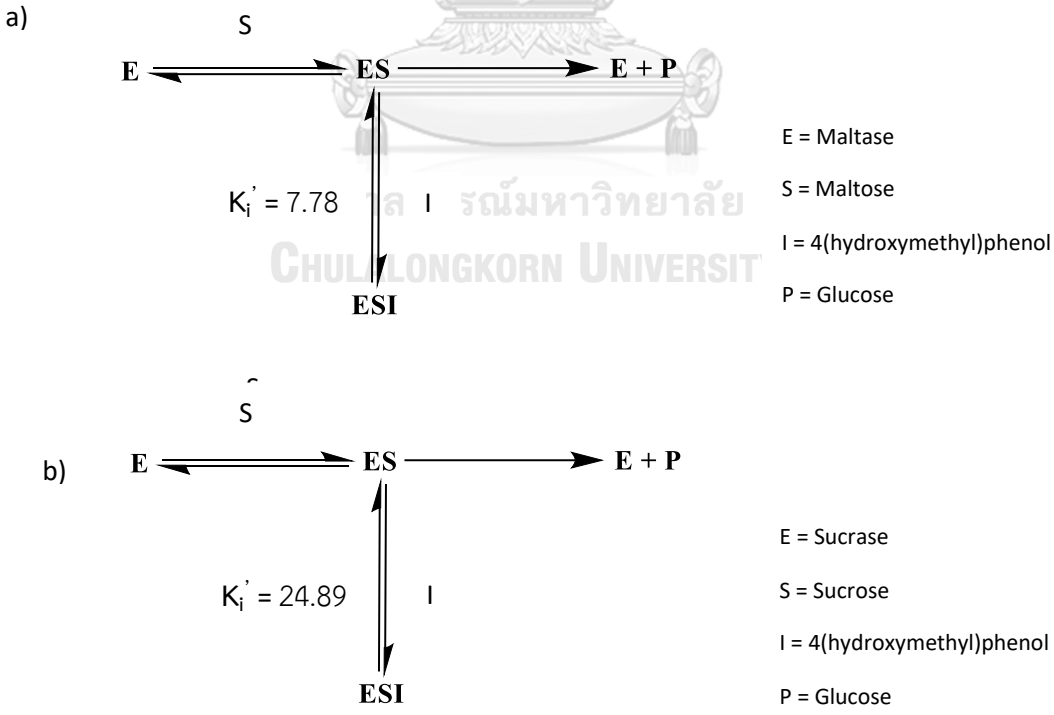


Figure 36 Putative mechanism pathway of 4(hydroxymethyl)phenol for uncompetitive inhibition against maltase(a) and sucrase(b)

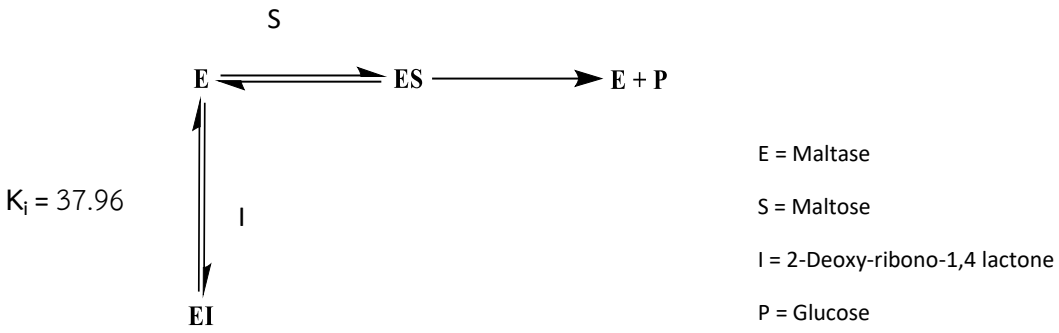


Figure 37 Putative mechanism pathway of 2-deoxy-ribono-1,4 lactone for competitive inhibition against maltase

In terms of inhibition against α-glucosidase, 5-HMF had no effect, but HMFCA, 4-(hydroxymethyl) phenol, and 2-deoxy-ribono-1,4 lactone were active. One point of interest in this experiment was the slightly altered structure of 5-HMF to HMFCA, a conversion of functional groups from aldehyde to carboxylic. It was found that the inhibitory activity of α-glucosidase was significantly changed. Therefore, this is knowledge that may be applied to predict and improve the performance of α-glucosidase inhibitors in future work. Structural activity relationship (SAR) is the relation between chemical structure and biological activity. This information may be used for screening or drug development. In addition to the data on α-glucosidase inhibition in this experiment, there are also reports from previous studies that have discussed how biological activity changes when a substance molecule is changed from aldehyde to carboxylic acid, such as, in the research of Amborabe and coworker (2002) [43]. They evaluated the antifungal activity of chemical compounds, and the results showed that the chemical change from aldehyde in salicylaldehyde to carboxylic in salicylic acid greatly increased its activity. Moreover, the DPPH scavenging activity of the hydroxybenzaldehydes and hydroxybenzoic acids also shows different effects [44]. Furthermore, In 1990, Shih and colleague investigated the effects of R4 substitution of 6-fluoro-2-(cyclohexylphenyl-4-yl)-3-methylquinolines on dihydroorotate

dehydrogenase activity. The result showed that the carboxylic acid-replaced R4 had a better inhibitory effect against enzyme compared to the aldehyde-replaced R4 [45]. From previous reports, it was found that 5-HMF can be converted to HMFCA and many other substances in the same group depending on the conditions [46]. In addition to its inhibitory effect on **α**-glucosidase, HMFCA has also been reported to have an anticancer effect. Likewise, 4-(hydroxymethyl)phenol. It has been reported to have bioactive, anti-inflammatory, pain-relieving, and antioxidant properties. There have also been reports of cerebrovascular disease treatment in laboratory animals [47] [48].

Furthermore, the kinetic study revealed that HMFCA and 4-(hydroxymethyl)phenol inhibited maltase and sucrase enzyme function in an uncompetitive manner, indicating that the inhibitor does not bind to free enzymes but only to enzymes bound to the substrate. This type of action makes it harder for reactants to convert into products. Although in this experiment HMFCA and 4-(hydroxymethyl)phenol were not as powerful as the standard drug acarbose, they would be interesting models that may be helpful in planning how the medication works [49]. In case of HMFCA and 4-(hydroxymethyl)phenol, the increased substrate concentration has no effect on the inhibitory action, whereas the standard drug acarbose has a competitive inhibitory mechanism that may reduce its efficacy in high substrate concentrations. 2-Deoxy-ribono-1,4-lactone retards maltase in a competitive manner like acarbose. When an inhibitor is present, the enzyme binds to it rather than the substrate, but it has lower potential compared to acarbose, which is used as a standard drug because it binds to the active site of the enzyme with lower affinity. which, according to the result of inhibitory activity (table 1 and 2).

This research has shown that black garlic contains active compounds that have **α**-glucosidase inhibiting activity, which affects blood sugar control. This information may be helpful in treating diabetes or preventing its complications that may arise due to chronic hyperglycemia and the development of future diabetes drugs.

Chapter 4

Conclusion

Black garlic is currently a well-known health food with many medicinal properties claimed, one of which is diabetes. Although there are several studies on the antidiabetic effects of black garlic, the identification of the active compound responsible for the inhibitory mechanism has not been documented. To answer the question, this study is the first identification of **α**-glucosidase inhibitors from black garlic. Based on bioassay-guided solation, four compounds were isolated which include 5-hydroxymethyl furfural, 5-hydroxymethyl-2-furan carboxylic acid, 4 (hydroxymethyl) phenol, and 2-deoxy-ribono-1,4 lactone. A colorimetric method was used to evaluate the **α**-glucosidase inhibitory activity of isolated compounds. The result showed that 5-hydroxymethyl furfural has no inhibitory activity against **α**-glucosidase. However, 5-hydroxymethyl-2-furan carboxylic acid (HMFCA), 4-(hydroxymethyl)phenol, and 2-deoxy-ribono-1,4 lactone were active against maltase with IC_{50} values of 18.83 ±0.91, 23.70 ±1.52 mM, and 9.26±0.31, respectively. Meanwhile, HMFCA and 4-(hydroxymethyl)phenol were also active against sucrase with IC_{50} values of 17.42 ±0.84 and 6.78 ±0.17mM, respectively. Our findings also provided an insight into the mechanism of **α**-glucosidase inhibition by particularly active compounds using kinetic studies. The results indicated that 5-hydroxymethyl-2-furan carboxylic acid, and 4 (hydroxymethyl) phenol inhibit enzyme function in an uncompetitive manner in both maltase and sucrase. On the other hand, 2-deoxy-ribono-1,4 lactone was active against only maltase in a competitive manner. Unlike previous investigations mainly performed in animal models (in vivo), this study demonstrated that black garlic could control glycemia through inhibiting **α**-glucosidase function of hydrolyzing oligosaccharide. However, more research into another mechanism responsible for diabetes, as well as the identification of active compounds using alternative methods of isolation, are required to close the black garlic research

gap for provide information to consumers as a healthy food choice for the prevention and control of diabetes and its complications.

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