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Comparative analysis of *Bombyx batryticatus* and *Bombyx mori* on α-glucosidase inhibition and their bioactive compositions

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Abstract

This study aimed to evaluate and compare the inhibitory effects of extracts from *Bombyx batryticatus* (BBE) and *Bombyx mori* (BME) on α-glucosidase, DPP-4, and LDL oxidation activities, focusing on their potential applications in managing postprandial hyperglycemia and metabolic syndrome. The results demonstrated that both BBE and BME effectively inhibited α-glucosidase and LDL oxidation, with BBE exhibiting higher inhibitory activity than BME. HPLC analysis identified linolenic acid, linoleic acid, linolenic acid ethyl ester, pheophorbide a, and pyropheophorbide a as key compounds contributing to these effects. Notably, the identified unsaturated fatty acids and pheophorbides showed strong α-glucosidase inhibitory activity, surpassing that of acarbose, a standard diabetic drug. These results suggest that, in addition to the previously reported 1-DNJ and fibroin proteins, unsaturated fatty acids and chlorophyll-derived pheophorbides may play significant roles in glycemic control. Compounds, particularly those from BBE, present promising opportunities for the development of natural therapeutic agents for diabetes management. The study concludes that BBE and BME have strong potential as functional ingredients in future diabetes treatment strategies, possibly offering enhanced efficacy over conventional inhibitors.

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Introduction

By 2024, diabetes remains a growing global health challenge, affecting approximately 540 million people worldwide, with this number projected to increase to 783 million by 2045 (IDF, 2021 #99). In response to this escalating crisis, significant advancements have been made in diabetes treatment and management. Despite the development of novel drugs such as glucagon-like peptide 1 (GLP-1) receptor agonists, dipeptidyl peptidase 4 (DPP-4) inhibitors, and sodium-glucose cotransporter-2 (SGLT2) inhibitors, α-glucosidase inhibitors (AGIs) continue to occupy a unique and valuable position in the therapeutic landscape (Pan *et al.*, 2020). AGIs, such as acarbose, work by delaying the digestion of carbohydrates in the small intestine, thereby reducing postprandial blood glucose spikes, which is an important aspect of type 2 diabetes management (Rosak and Mertes, 2012).

AGIs have been shown to offer additional benefits beyond

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Table 1. Characteristics and ethanol extraction yield of BB and BM.

	Weight (g)	Length (cm)	Color	Water content (%)	Yield of ethanol extraction (%)
BB	0.6 ± 0.1	3.8 ± 0.5	Yellow, Brown	9.0 ± 0.1	9.0 ± 0.3
BM	0.6 ± 0.1	4.6 ± 0.3	Light yellow	8.6 ± 0.6	10.2 ± 0.4

glucose control. Studies suggest that they may help modulate gut microbiota and reduce inflammatory markers, contributing to broader metabolic advantages (Zhang *et al.*, 2019). AGIs can be particularly effective for managing postprandial glucose levels for populations with high-carbohydrate diets, such as those in East Asia (Khwaja and Arunagirinathan, 2021). In the current era of personalized medicine, the ability to tailor treatments to individual patient needs, especially for those who may benefit more from targeting postprandial glucose, ensures that AGIs remain a valuable tool in the comprehensive management of diabetes (Chen *et al.*, 2022; Tian *et al.*, 2023).

Silkworms, scientifically known as *Bombyx mori* (BM), have been utilized in traditional medicine across various cultures, especially in East Asia (Ma *et al.*, 2022). Silkworm pupae are rich in proteins, fatty acids, and other nutrients, and they have been reported to possess several health benefits, including antioxidant, anti-inflammatory, hypoglycemic, and anticancer activities (Zhou *et al.*, 2022). *Bombyx batryticatus* (BB) is a silkworm that has died and become stiff due to infection by the entomopathogenic fungus *Beauveria bassiana*. BB is considered a promising source of bioactive compounds with significant potential in treating epilepsy, preventing thrombosis, and protecting against oxidative stress-related neuronal damage (Hu *et al.*, 2019; Hu *et al.*, 2017). It is also traditionally used for its unique bioactive properties, particularly in neuroprotection and platelet aggregation inhibition (He *et al.*, 2020; Kong *et al.*, 2014). In contrast, BM, studied in its larval and pupal stages, contains different bioactive compounds such as 1-deoxynojirimycin (1-DNJ), antimicrobial peptides, and antioxidants, which primarily focused on metabolic health and immune support (Wang *et al.*, 2021; Wang *et al.*, 2012).

Several studies have reported that BB, which also contains 1-DNJ as an active ingredient, can improve diabetes, one of the most common metabolic diseases (Jeong *et al.*, 2004; Zhao *et al.*, 2018). In addition to 1-DNJ, other compounds present in BM and BB, such as unsaturated fatty acids and chlorophyll derivatives like pheophorbides, may also contribute to their α-glucosidase inhibitory activity. However, a direct comparison of the inhibitory effects of BM and BB extracts on key metabolic enzymes, including α-glucosidase and DPP-4, as well as their ability to inhibit lipid peroxidation, has not been extensively explored. This study aims to fill this gap by evaluating and comparing the in vitro effects of *Bombyx mori* extract (BME) and *Bombyx batryticatus* extract (BBE) on α-glucosidase, DPP-4, and LDL oxidation activities. The study also seeks to identify the active compounds responsible for these activities and assess their potential as functional ingredients for diabetes management. Through these investigations, we hope to provide insights into the therapeutic potential of BM and BB and explore the possibility of developing new dietary supplements or therapeutic agents that harness these bioactive compounds.

Methods and materials

Chemicals and reagents

Linolenic acid, linoleic acid, and linolenic acid ethyl ester were purchased from Sigma-Aldrich (Saint Louis, MO, USA), with a purity of 99%. Pheophorbide a and pyropheophorbide a were obtained from Cayman Chemical (Ann Arbor, MI, USA) and Toronto Research Chemicals (Toronto, ON, Canada), respectively. Phosphate and Tris buffer were obtained from LPS Solution (Daejeon, Korea).

Extracts of *Bombyx batryticatus* and *Bombyx mori*

Dried *Bombyx batryticatus* (BB) and *Bombyx mori* (BM) were purchased from Seoul Gyeongdong Market (Seoul, Korea) and ground into a fine powder. A total of 500 mg of the grounded powders of BB and BM were extracted using 5 mL of 95% ethanol for 24 h with shaking at 25 °C. After centrifugation, the supernatant was evaporated to dryness under reduced pressure, yielding 45 mg of BBE and 51 mg of BME. Table 1 provides detailed characteristics of the BB and BM samples, including their weight, length, color, water content, and ethanol extraction yield.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined according to a modified method from a previous study (Kim *et al.*, 2008). The reaction conditions comprised 2.5 mM *p*-nitrophenyl α-D-glucopyranoside and 0.1 U/mL α-glucosidase in 0.1 M phosphate buffer (pH 6.8). A 5 μL sample solution was added to initiate the reaction. Each reaction was carried out at 37 ℃ for 30 min and was stopped by adding 100 μL of 0.1 M Na_2CO_3. Enzymatic activity was quantified by measuring absorbance at 405 nm. The IC_{50} value was defined as the concentration of the α-glucosidase inhibitor that inhibited 50% of α-glucosidase activity. Acarbose (Sigma-Aldrich), an α-glucosidase inhibitor, was used as a positive control.

DPP-4 inhibitory activity

The method for measuring DPP-4 enzyme activity was conducted as previously described (Kim *et al.*, 2005). The enzyme activity of human recombinant DPP-4 (Prospec-Tany Technogene Ltd., NJ, USA) was measured using H-Ala-Pro-AFC (AnaSpec co., CA, USA) as a substrate. Briefly, the reaction conditions comprised 0.1 μg/mL DPP-4 and 40 μM H-Ala-Pro-AFC in 50 mM Tris buffer (pH 7.5). The samples were dissolved in dimethyl sulfoxide and added to the reaction mixture for 60 min at 25 ℃. AFC, as an indicator of DDP-4 activity, was detected using a Fluorometer Victor 2 (Perkin Elmer, MA, USA) at an excitation of 355 nm and emission of 510 nm. Sitagliptin (MedChemExpress, Shanghai, China), a DPP-4 inhibitor, was used as a positive control.

Lipid peroxidation inhibitory activity

The low-density lipoprotein (LDL)-oxidation activity was measured using thiobarbituric acid reactive substance (TBARS) assay, as reported previously (Ji *et al.*, 2019). Briefly, 230 μL of LDL solution (Invitrogen, Carlsbad, CA, USA) was incubated with 10 μL of extracts and 10 μL of 125 μM $CuSO_4$ at 37°C for 4 hours to induce oxidation. After incubation, 1 mL 20% trichloroacetic acid and 1 mL 0.67% 2-thiobarbituric acid were added to stop the reaction and precipitate the mixture. The reaction mixture was then heated for 10 min at 95 ℃, cooled on ice, and centrifuged for 15 min at 1500 *g*. The optical density of the collected supernatant was measured at 532 nm. Butylated hydroxytoluene (BHT), an antioxidant, was used as a positive control, and malondialdehyde bis(diethyl acetal) was used as a standard.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed to identify the active components of BBE and BME that exhibit α-glucosidase inhibitory activity. The components of BBE and BME were analyzed using a HPLC-diode array detector system (Shimadzu Corp., Tokyo, Japan). The extracts were separated on a Brownlee SPP C18 column (4.6 × 50 mm, 2.7 μm; Perkin Elmer, Inc., Waltham, MA, USA). The mobile phase compositions were 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B). The linear gradient elution program was as follows: 5–50% B at 0–15 min, 50–100% B at 15–20 min, 100% B at 20–25 min, 100–5% B at 25–27 min, and 5% B at 27–30 min. The absorbance of the HPLC profile was 210 nm and 410 nm, with a flow rate of 1.8 mL/min.

Purchased compounds linolenic acid, linoleic acid, linolenic acid ethyl ester, pheophorbide a, and pyropheophorbide a were used as standards, and serial dilutions of each compound were plotted against the HPLC peak area to generate calibration curves for the four individual compounds. The regression equations from those standard curves were then used to quantify (mg/g extract) the four individual compounds in BBE and BME.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Significant differences among the groups were assessed by Student's *t*-test, using JMP software (SAS Institute Inc., Cary, NC, USA), and p value < 0.05 was considered significant.

Results and Discussion

Effects of BBE and BME on enzyme activity comparison

Measuring the activities of α-glucosidase, DPP-4, and lipid peroxidation provides crucial insights into their potential therapeutic benefits and biological impacts on metabolic syndrome (Scheen *et al.*, 2015; Masenga *et al.*, 2023). Initially, we compared the *in vitro* enzyme activities of BBE and BME on α-glucosidase, DPP-4, and LDL-oxidation. Treatment with both BBE and BME effectively inhibited α-glucosidase activity in a dose-dependent manner, with mild inhibitory effects observed on DPP-4 activity (Table 2). Both BBE and BME effectively inhibited LDL oxidation. When comparing the

Table 2. α-Glucosidase, DPP-4, and LDL-oxidation inhibitory activities of BBE and BME.

	α-Glucosidase inhibition (%)			DPP-4 inhibition (%)	LDL-oxidation inhibition (%)
	250 μg/mL	25 μg/mL	5 μg/mL	200 μg/mL	40 μg/mL
BBE	99.1 ± 0.1**	95.1 ± 1.1**	49.1 ± 2.4**	22.4 ± 1.2**	64.3 ± 2.0**
BME	89.7 ± 0.9	84.2 ± 1.0	29.6 ± 1.7	14.1 ± 2.1	46.2 ± 2.9
Positive control	52.7 ± 0.7 (Acarbose, 0.5 mM)			49.8 ± 2.0 (Sitagliptin 25 nM)	50.3 ± 2.1 (BHT 2 μM)

Values indicate mean ± SD (n = 3).
** $p < 0.01$ compared to the BME group.

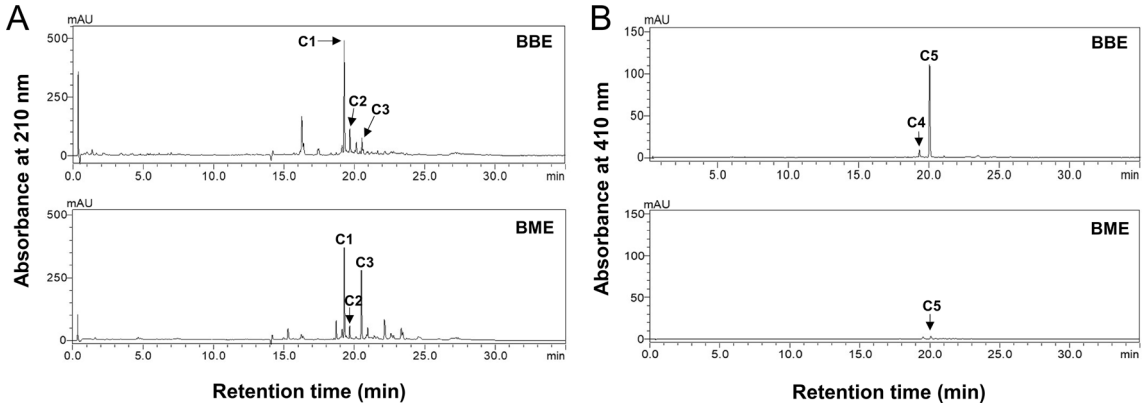

Fig. 1. HPLC profiles of BBE and BME. HPLC chromatograms of BBE and BME were detected at 210 nm (A) and 410 nm (B).

activities of BBE and BME, BBE exhibited higher inhibitory activity than BME against both α-glucosidase and LDL oxidation (Table 2).

These results are consistent with previously reported anti-diabetic and antioxidant activities of BM and BB (Hu *et al.*, 2017; Zhao *et al.*, 2018; Zhou *et al.*, 2022). The α-glucosidase inhibitory activity of BM has been primarily attributed to 1-DNJ (Ju *et al.*, 2015; Rattana *et al.*, 2019). Generally, the 1-DNJ content is known to decrease during the metamorphosis of BM into BB (Chen, 2014). In this study, BBE exhibited higher α-glucosidase inhibitory activity than BME, suggesting that BBE may contain other active substances besides 1-DNJ that contribute to its α-glucosidase inhibitory effect. The potent α-glucosidase inhibitory activity of both BBE and BME indicates their potential use as functional ingredients.

Compounds of BBE and BME

The HPLC profiles showed that BBE and BME contained compounds C1–C3 at 210 nm and compounds C4 and C5 at 410 nm (Fig. 1). The chemical structures of compounds C1–C5 were identified as linolenic acid (C1), linoleic acid (C2), linolenic acid ethyl ester (C3), pheophorbide a (C4), and pyropheophorbide a (C5) by comparison with their respective standards (Fig. 2). Furthermore, the content of these compounds was measured in both BBE and BME. As shown in Table 3, linolenic acid, linoleic acid, and pyropheophorbide a were more abundant in BBE than

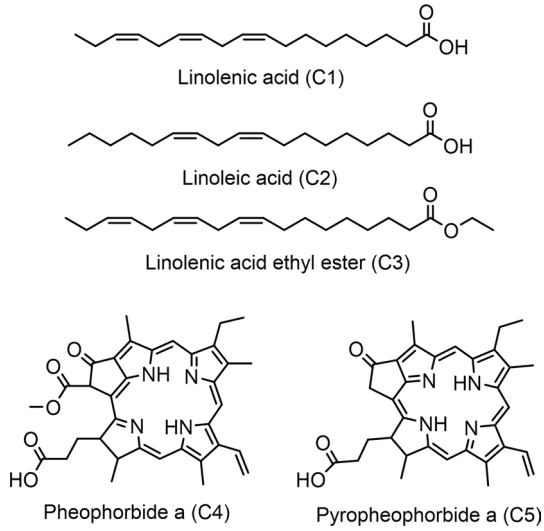

Fig. 2. Chemical structures of the main components. The chemical structures of linolenic acid (C1), linoleic acid (C2), linolenic acid ethyl ester (C3), pheophorbide a (C4), and pyropheophorbide a (C5) were confirmed in BBE and BME.

Table 3. Content of compounds from BBE and BME.

	BBE	BME
Linolenic acid (mg/g Ex.)	79.1	48.8
Linoleic acid (mg/g Ex.)	30.4	12.2
Linolenic acid ethyl ester (mg/g Ex.)	6.4	35.1
Pyropheophorbide a (mg/g Ex.)	0.3	ND

ND, not detected (lower than detection limit).

in BME, while linolenic acid ethyl ester was more abundant in BME than in BBE.

Interestingly, both BBE and BME, which showed effective α-glucosidase inhibitory activity, did not contain 1-DNJ, possibly due to the predominant extraction of fatty acids using 95% ethanol as the solvent. Various fats and fatty acids, including both saturated and unsaturated fatty acids, are also present in BB and BM (Hu *et al.*, 2017; Park *et al.*, 2014). During the transformation of BM into BB, fats are hydrolyzed into fatty acids by lipases from *Beauveria bassiana* (Xing *et al.*, 2019). Similarly, this study found that the levels of fatty acids, including linolenic acid and linoleic acid, were higher in BBE than in BME. Previously, 1-DNJ and fibroin have been identified as the main active ingredients in BM and BB for glycemic control (Zhou *et al.*, 2022). The results of this study suggest that, in addition to these components, unsaturated fatty acids and pheophorbides may also contribute to glycemic control. To confirm this, we measured the α-glucosidase inhibitory activity of the compounds identified in BB and BM.

Effects of BBE and BME compounds on α-glucosidase activity

The α-glucosidase activity of each compound was assessed at a concentration of 10 μM. The results indicated that all compounds exhibited stronger α-glucosidase inhibitory activity than acarbose (Table 4). Linolenic acid demonstrated moderate inhibition, with 34.2% inhibition at 10 μM and an IC_{50} value of 15.1 μM, whereas linoleic acid showed significantly higher inhibitory activity, with 62.2% inhibition at 10 μM and an IC_{50} of 6.1 μM. Linolenic acid ethyl ester, a derivative of linolenic acid, exhibited the highest α-glucosidase inhibition among the fatty acids, achieving 72.9% inhibition at 10 μM and an IC_{50} of 4.6 μM. The esterification of linolenic acid appears to enhance its inhibitory potency, possibly by improving its interaction with the enzyme or increasing its solubility. Pheophorbide a,

Table 4. α-Glucosidase inhibitory activity of compounds from BBE and BME.

	α-Glucosidase inhibition (%)	
	10 μM	IC_{50} (μM)
Linolenic acid (C1)	34.2 ± 2.1	15.1
Linoleic acid (C2)	62.2 ± 1.7	6.1
Linolenic acid ethyl ester (C3)	72.9 ± 0.9	4.6
Pheophorbide a (C4)	65.0 ± 1.7	4.5
Pyropheophorbide a (C5)	86.0 ± 1.8	1.8
Acarbose	55.4 ± 0.7 (0.5 mM)	-

derived from chlorophyll degradation, proved to be a highly effective α-glucosidase inhibitor. Pyropheophorbide a exhibited the highest inhibition, achieving 86.0% inhibition at 10 μM and the lowest IC_{50} of 1.8 μM among all tested compounds. These findings suggest that both fatty acids and chlorophyll-derived pheophorbides from BBE and BME have strong potential as α-glucosidase inhibitors, with promising applications in managing postprandial blood glucose levels in diabetic patients.

Linolenic acid and linoleic acid are essential fatty acids that play crucial roles in human health. These fatty acids cannot be synthesized by the human body and must be obtained through the diet. Both of these essential fatty acids are primarily known for their benefits to cardiovascular health and their anti-inflammatory properties (Du *et al.*, 2024). Consistent with our results, many fatty acids, including linolenic acid and linoleic acid, have been shown to exhibit strong anti-α-glucosidase activity and act as competitive inhibitors (Su *et al.*, 2013). Ethyl esters of fatty acids have been studied primarily in the context of long-chain omega-3 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Some studies suggest that the triglyceride form of these fatty acids is better absorbed than the ethyl ester form, while others have found no significant difference or even superior absorption with ethyl esters under certain conditions (Offman *et al.*, 2017; Schuchardt and Hahn, 2013). However, no studies have directly compared the α-glucosidase inhibitory activity of unsaturated fatty acids and their ethyl ester forms. This study suggests that linolenic acid ethyl ester exhibits enhanced α-glucosidase inhibitory activity compared to linolenic acid.

Pheophorbide a, isolated from the red algae *Gelidium amansii*, exhibited a strong inhibitory effect on α-glucosidase activity, partly attributable to the interaction between the enzyme and

the hydroxyl group of pheophorbide (Kim *et al.*, 2019), which resulted in a pronounced reduction in postprandial blood glucose levels (Kim *et al.*, 2019; Li *et al.*, 2015). Consistent with these reported studies, pheophorbide a and its derivative, pyropheophorbide a, also exerted a strong inhibitory effect on α-glucosidase activity. Therefore, pheophorbide a and pyropheophorbide a are promising candidates for future development as natural therapeutic agents for diabetes management and the prevention of complications. In summary, this study demonstrates the potential of BB and BM compounds to outperform conventional inhibitors such as acarbose, suggesting that BB and BM may play an important role in future diabetes treatment strategies.

Conclusion

The study demonstrated that *Bombyx batryticatus* and *Bombyx mori* possess significant α-glucosidase and LDL-oxidation inhibitory activities, highlighting their potential as natural therapeutic agents for managing diabetes and metabolic syndrome. BBE, in particular, exhibited superior inhibitory activity compared to BME, which could be attributed to its higher content of bioactive compounds such as linolenic acid, linoleic acid, and pyropheophorbide a. The enhanced α-glucosidase inhibitory effect observed in linolenic acid ethyl ester, along with the strong inhibition by pheophorbide a and pyropheophorbide a, suggests that these compounds could outperform traditional inhibitors like acarbose in controlling postprandial blood glucose levels. These findings not only expand our understanding of the bioactive components in BBE and BME but also underscore their potential in developing new dietary supplements or therapeutic agents for diabetes management. Future research should explore the clinical applications of these findings and further investigate the molecular mechanisms underlying the observed bioactivities.

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