

α -GLUCOSIDASE AND TYROSINASE INHIBITORY GUIDED ISOLATION, IDENTIFICATION OF MAJOR COMPOUND AND IN SILICO STUDY FROM EDIBLE MUSHROOM *COPRINUS COMATUS*

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Abstract

Coprinus comatus is an edible and medicinal mushroom. The crude hexane, EtOAc and methanol extracts were extracted successively from *C. Comatus*. All the crude extracts were evaluated for anti- α -glucosidase and tyrosinase. The results showed that %inhibition valued for the crude hexane, EtOAc and methanol extracts of anti- α -glucosidase were 98.58 ± 0.09 , 98.90 ± 0.21 , and 6.68 ± 0.79 , respectively. The anti-tyrosinase for the crude hexane, EtOAc and methanol extracts were 97.31 ± 2.77 , 97.31 ± 3.34 and 64.94 ± 3.34 , respectively. Notably, the bioassay-guided showed that the crude hexane and EtOAc were the highest for anti- α -glucosidase and tyrosinase activities, which led to the interest in purifying the major compound. The isolation and purification were successively done using chromatographic techniques. The pure compounds were determined by spectroscopic analysis (UV, FTIR, and NMR) and comparison with previously reported. The isolation led to three compounds (1-3), ergosterol (1), indole-3-carbaldehyde (2), and 4-hydroxybenzoic acid (3). The molecular docking of compounds 1-3, reported as α -glucosidase and tyrosinase inhibitors, was done by the AutoDockTools 1.5.6 (ADT) software. The results revealed that ergosterol (1) provides the highest binding free energy (-9.91 kcal/mol) to the active site of α -glucosidase, indicating that it is quite specific to this enzyme. Moreover, ergosterol (1) has strong binding energy (-9.64 kcal/mol) to tyrosinase but not significant tyrosinase specificity. This evidence supports that *C. Comatus* would be applicable for a functional food for controlling glucose blood levels.

Keywords: Diabetes mellitus; Hyperpigment; Molecular docking; Tyrosinase; α -glucosidase

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Introduction

Mushrooms are one of the important food sources for humans. These are essential dietary supplements that are critical to both health and disease. Their polyunsaturated fat content is low. Consequently, consuming mushrooms aids in weight loss, the same as a diet high in mannitol, low in fat and low in glucose, which can prevent diabetes (Omale *et al.*, 2020). Moreover, mushrooms are widely used in medicine, pharmacy, and cosmetics. Mushrooms are functional food due to their source of bioactive compounds and have numerous nutritional values (Hossain *et al.*, 2021). Many species of mushrooms were studied as anti-cancer agents, and they show great anticancer potential (Abiodun *et al.*, 2022). In addition, the mushrooms showed antifungal activity, antimicrobial activity, neuroprotective properties, anti-tyrosinase activity and anti-Type-2 diabetes (T2DM) activity (Hyder and Dutta, 2021; Agunloye and Oboh, 2022). Mushrooms are a natural food source of vitamin D, (Pinto *et al.*, 2020). Mushrooms are valuable raw materials for a variety of culinary uses, such as unique functional and nutritional attributes, phytochemicals, partial meat and starch replacement, fermentation substrate, protein-rich products, and cosmetic products (Mwangi *et al.*, 2022).

The enzyme tyrosinase causes hyperpigmentation in human skin and browning of fruits, fungi, and vegetables. This metalloenzyme regulates melanin synthesis, a general word for a class of naturally occurring pigments seen in plants, animals, and humans. Melanin is the pigment that gives human skin its color, but it also plays a role in the enzymatic browning of freshly cut fruits and vegetables (Zolghadri *et al.*, 2019; Carcelli *et al.*, 2020;). Tyrosinase inhibitors may, therefore, be useful as antibrowning chemicals for the food and beverage industries in addition to being skin-whitening compounds (You *et al.*, 2022).

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia (high blood glucose levels) due to a defect in insulin secretion and a certain amount of peripheral insulin resistance that cannot be avoided. Hyperglycemia and a host of other problems, including hyperlipidemia and atherosclerosis, are brought on by diabetes mellitus, a progressive metabolic disorder characterized by abnormalities in glucose, lipids, and lipoproteins (Rizzo *et al.*, 2021). DM is classified into three types: Type I diabetes arises due to the inability of pancreatic β -cells to make insulin, a typical occurrence in children and teenagers; Type 2

diabetes develops because of insulin resistance or the body's cells becoming insensitive to insulin, whereas gestational diabetes develops during pregnancy (Srivastava *et al.*, 2020). T2DM accounts for approximately 90% of cases of diabetes worldwide (Zolghadri *et al.*, 2019). α -Amylase and α -glucosidase are sugar-hydrolyzing complex enzymes that are the leading cause of T2DM. So, inhibiting these enzymes can reduce glucose blood levels for T2DM therapy (Zolghadri *et al.*, 2019). The effects of diabetes include a shortened life expectancy, considerable morbidity from particular microvascular and macrovascular problems associated with the disease, and a lowered standard of living.

C. comatus is an edible and medicinal mushroom found in Asia, Europe, the Americas, and Australia (Gao *et al.*, 2021). The *C. comatus* is also known as chicken drumstick mushroom, shaggy ink cap, lawyer's wig, shaggy mane and Thailand, as hed-kone-noy. Interestingly, *C. comatus* has been shown to benefit glycemic control in diabetes with much research in the past decade (Ratnaningtyas *et al.*, 2022; Dimopoulou *et al.*, 2023). Moreover, *C. comatus* reported that anticancer, antioxidant and rich source of polysaccharides and nutrients (Emsen and Guven, 2020; Stilinović *et al.*, 2020; Gao *et al.*, 2021). To this, *C. comatus* possesses a variety of pharmacological activities and is a good source of nutrition, so it is interesting as a functional food (Dimopoulou *et al.*, 2023).

In Thailand, *C. comatus* was first developed and cultivated in 2014 for new economic mushroom strains (Chaiyama *et al.*, 2007). Although antidiabetic from *C. comatus* has been reported in the previous literature, in Thailand, has not been reported. *C. comatus* should be studied in other biological activities, such as anti-tyrosinase. That's the cause of hyperpigmentation in human skin to develop this mushroom into another product, not only as a functional food and has not been reported yet in this activity.

Herein, we study anti- α -glucosidase, which is one cause of diabetes mellitus and anti-tyrosinase, which causes hyperpigmentation in human skin. Moreover, we studied the major compounds in *C. comatus* cultivated in Thailand to understand the active component and in silico study to know the mechanism of pure compounds to their activity. This information is a database for developing a new economic mushroom strain (*C. comatus*), which was grown in Thailand and gave rise to the possibility of expanding these mushrooms into various products.

Materials and Methods

General experimental procedures

The NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer using CDCl_3 and CD_3OD or a mixture of both solvents. The internal standards were referenced from the residual peak of CDCl_3 and CD_3OD , and which operated at 400 MHz (^1H) and 100 MHz (^{13}C). IR spectra were obtained using a Bruker INVENO spectrophotometer. UV spectra were recorded using a Shimadzu UV-1800 apparatus (Japan). MERCK silica gel 60 F254 TLC aluminum sheets were used for thin layer chromatography (TLC) experiments. Silica gel measuring between 0.063 and 0.200 mm or smaller (Darmstadt, Germany) and Sephadex LH-20 were used for column chromatography. Preparative thin layer chromatography (PTLC) was carried out on glass-supported silica gel plates using silica gel 60 PF₂₅₄ for preparative layer chromatography (Darmstadt, Germany). All solvents were routinely distilled before use. The α -Glucosidase enzyme type I was from *Saccharomyces cerevisiae*, *p*-Nitrophenyl α -D-glucopyranoside, acarbose, Kojic acid, L-tyrosine, and L-DOPA (L-3,4-dihydroxyphenylalanine) were purchased from Sigma-Aldrich (USA).

Plant materials

The cultivated *C. comatus* was purchased from Wanon Niwat District, Sakon Nakhon Province, Thailand, in February 2023. A voucher specimen (SNRU012023) was deposited at the Department of Chemistry, Faculty of Science and Technology, Rajabhat Sakon Nakhon University, Thailand.

Extraction and isolation

The fruiting bodies of *C. comatus* (3.0 kg) were ground and extracted with Hexane, EtOAc and MeOH (3 $\times$ 2 L, 72 h each) at room temperature to yield a crude Hexane (8.0 g), EtOAc (46.9 g), and MeOH (48.6 g) extracts by evaporating the solvent with a rotary evaporator under reduced pressure. The crude hexane and EtOAc extract were selected for isolation due to their higher activities than MeOH extracts, based on bioassay-guided assay.

The crude hexane extract (8.0 g) was subjected to column chromatography (CC) using 60 - 120 mesh silica gel and eluted successively with varying concentrations from 0 to 40% EtOAc and hexane. The eluted fractions were collected in 200 mL flasks, and they were pooled based on the similarity of their TLC spots, leading to obtaining a total of eight fractions (H1 - H8). Fraction 7 was crystallized with MeOH to give compound 1 (2.4 g).

The crude EtOAc extract (46.9 g) was subjected to 60 - 120 mesh silica gel CC (20 - 80 % EtOAc-hexane and 10 - 50 % MeOH-EtOAc) to provide five fractions (E1 - E5). Fraction E3 (101.4 mg) was subjected to Sephadex LH-20 CC (MeOH) to give 4 subfractions (E3.1-E3.4). Subfraction E3.3 was re-crystallized with MeOH to obtain compound 2 (1.6 mg). Re-chromatography of fraction E3.4 (22.8 mg) was performed using Sephadex LH-20 CC (MeOH) to give 2 subfractions (E3.4.1-E3.4.2). Compound 3 (1.3 mg) was obtained from subfraction E3.4.1 by re-crystallized with MeOH.

α -Glucosidase inhibitory activity assay

The protocol for inhibition of α -glucosidase activity using a procedure from the literature with minor modifications of Kittiwisut *et al.* in 2021 (Kittiwisut *et al.*, 2021). A 1 mg/mL extract solution was obtained by dissolving each separate crude extract in 0.1 M sodium phosphate buffer (pH 6.8). A 96-well plate containing 50 μL of 1 mM *p*-nitrophenyl- α -D-glucopyranoside was added with 50 μL of sample solution. After five minutes at 37°C, 50 μL of 0.05 U/ml of α -glucosidase enzyme in buffer was added to each well of the sample mixtures. The reaction was preincubated at 37°C for 20 min. Then, the reaction was stopped using 50 μL of 0.3 M sodium carbonate solution. The absorbance was measured at 405 nm using a BioRAD Benchmark Plus™ microplate reader (USA). The percentage of enzyme inhibitory effect was calculated and compared to the that of α -glucosidase inhibitor (acarbose). The following equation calculated the percentage inhibition of the α -glucosidase activity:

$$\% \text{inhibition} = [((A-B)-(C-D))/(A-B)] \times 100$$

where A is an initial velocity of the control reaction with enzyme (control), B is an initial velocity of the control reaction without enzyme (control blank), C is an initial velocity of the enzyme reaction with sample and D is an initial velocity of the reaction with sample but without enzyme (sample blank).

Tyrosinase inhibitory activity assay

The previously modified method was used to assess the tyrosinase inhibitory activity in a 96-well microplate (Masuda *et al.*, 2005). The inhibitory activity of any substance was measured using L-DOPA as a substrate. Briefly, each reaction mixtures were placed in a total of eight wells, which were designated for A (three wells), B (one well), C (three wells), and D (one well): A: 120 μL of a 0.1 M phosphate buffer (pH 6.8) and 40 μL of tyrosinase

(100 units/ml) in the same buffer; B: 160 µl of the same buffer; C: 80 µl of the same buffer, 40 µl of tyrosinase (100 units/ml) in the same buffer, and 40 µl of an appropriate quantity of the sample-buffer solution containing 5% DMSO to dissolve the sample; D: 120 µl of the same buffer and 40 µl of the same amount of the sample solution containing 5% DMSO. After mixing the substances of each well and incubating for 20 minutes at 37°C, 2.5 mM of L-DOPA was added in 40 µl of the same buffer (Table 1). The absorbance at 490 nm for each well was measured following a 20 min incubation period at 37°C. The following equation calculated the percentage inhibition of the tyrosinase activity:

$$\% \text{inhibition} = [((A-B)-(C-D))/(A-B)] \times 100$$

where A is an initial velocity of the control reaction with enzyme (control), B is an initial velocity of the control reaction without enzyme (control blank), C is an initial velocity of the enzyme reaction with sample and D is an initial velocity of the reaction with sample but without enzyme (sample blank).

Table 1. The solution for Tyrosinase inhibitory activity assay

Solution	Buffer pH 6.8 (µl)	Tyrosinase (µl)	Sample (µl)	L-DOPA (µl)
A	120	40		40
B	160			40
C	80	40	40	40
D	120		40	40

Molecular docking

The three-dimension structures isolated from edible mushroom were produced from *C. comatus*, then were fully optimized by b3lyp/6-31G(d) level using Gaussian 09 program (Gaussian 09 (Revision B.01)) and converted to protein databank (pdb) format. The protein targets were downloaded from protein data bank (<http://www.pdb.org>); Alpha-glucosidase (PDB ID; 3TOP) and Tyrosinase (PDB ID; 2Y9X) (Ismaya *et al.*, 2011). All water molecules and non-interacting ions were removed. All missing hydrogen/side chain atoms were added using AutoDockTools 1.5.6 (ADT) (Morris *et al.*, 2009). Gasteiger charges were employed and all non-polar hydrogens were merged into carbon atoms (Gasteiger and Marsili, 1978). The grid box was set to 40, 40, and 40 grid points in the x, y, and z dimensions with a grid spacing of 0.375 Å. The energy grid maps were generated by AutoGrid 4.2.6 program (<http://autodock.scripps.edu/>). All docking calculations were performed by using AutoDock 4.2.6 program with Lamarckian genetic algorithm (LGA) searches of 200 runs, 2.5×10⁶ energy evaluations, and default values for other

parameters (Morris *et al.*, 1998). Discovery Studio Visualizer 2020 was used to visualize the protein-ligand interactions.

Results and Discussion

Extraction of *Coprinus comatus*

The dried fruiting bodies of *C. comatus* (3.0 kg) were extracted by the maceration technique, initially extracted from non-polar to high polar solvents (hexane, EtOAc and methanol). The crude extracts were classified as non-polar compounds or high polar compounds. The yield percentage of crude extract was in the range of 0.27-1.62 g/100 g dry weight mushrooms, as shown in Table 2.

Table 2. Extracted yield of *C. comatus*

Fractions	Characteristics of evaporated residue	Crude extract (g)	Extract yield (%)
Hexane	Yellow oil	8.01	0.27
EtOAc	Viscous brown	46.91	1.56
MeOH	Viscous brown	48.56	1.62

α -Glucosidase and Tyrosinase inhibitory activities of crude extract

Although *C. comatus* has reported many biological activities, such as anti- α -glucosidase from EtOH extract (Ratnaningtyas *et al.*, 2022) and tyrosinase, its component has not yet been identified. Therefore, bioassay-guided isolation together with the extraction techniques were performed to screening the major components with biological response-linked (Phukhatmuen *et al.*, 2020). This study tested all crude extracts on α -glucosidase and tyrosinase (Table 3).

Table 3. α -Glucosidase and Tyrosinase inhibitory activities of crude extract

Crude extract	% inhibition (SD, n = 3)	
	α -Glucosidase*	Tyrosinase**
Hexane	98.58 ± 0.09	97.31 ± 2.77
EtOAc	98.90 ± 0.21	97.31 ± 3.34
MeOH	6.68 ± 0.79	64.94 ± 3.34
Acarbose	73.26 ± 1.94	-
Kojic acid	-	96.93 ± 0.67

* α -Glucosidase inhibitory activity of crude extract (1 mg/ml).

** Tyrosinase inhibitory activity of crude extract (10 mg/ml).

The crude hexane and EtOAc extracts showed to have the strongest inhibition effect on α -glucosidase and tyrosinase activities. The % inhibition of α -glucosidase by crude hexane and EtOAc extracts, for which concentrations were at 1 mg/mL, were 98.58 ± 0.09 and 98.90 ± 0.21, respectively and also showed higher than that of the Acarbose, as the positive control. Moreover, The % inhibition of tyrosinase of crude hexane and EtOAc extracts were 97.31 ± 2.77 and 97.31 ± 3.34,

respectively, and also showed a stronger potent inhibitory effect near Kojic acid as a positive control, which all compounds were tested at the final concentration of 10 mg/mL. These results suggested that there are some compounds in both crude hexane and EtOAc extracts may possess these biological activities.

Major compound of crude hexane and EtOAc extracts

The crude hexane and EtOAc extracts of *C. comatus* significantly inhibited to α -glucosidase and tyrosinase activities. Therefore, crude hexane and EtOAc extracts were chosen for further purification using chromatography techniques. The chemical structures of the pure compounds were elucidated by spectroscopic methods. Their data of each compound were compared with those previously reported.

An isolated compound from crude hexane extract was obtained in large amounts as a white crystal solid, and the melting point is 150.6 - 153.9 °C. Firstly, we observed the functional group of the pure compound by the FT-IR spectrum in the range 4000 - 400 cm^{-1} , peaks at wavenumbers 3365 cm^{-1} corresponding to O-H stretching and C-H stretching at wavenumbers 2953 - 2869 cm^{-1} . The peaks at 2656 - 1368 cm^{-1} correspond to C=C. In 2007, Manea *et al.* also reported a similar pattern (Manea *et al.*, 2007). Moreover, comparing the FT-IR spectrum with the FT-IR libraries database revealed these spectrum results identical to ergosterol. This compound was intensively identified by ^1H and ^{13}C NMR spectra to confirm ergosterol structure.

^1H NMR (400 MHz, Chloroform- d) spectra (Figure 2(a)) confirmed sterol $\Delta^{5,7}$ structure by signals δ_{H} 5.57 (dd, $J = 2.2$; 5.5 Hz) and 5.38 (dd, $J = 2.6$; 5.4 Hz) diagnostic for olefin hydrogens H-6 and H-7, besides multiplet in δ_{H} 3.63 (H-3) indicate the presence of hydrogen linked to carbinolic carbon. Double bonds were observed at signal 5.21 (m) relative to H-22 and H-23. Still, signals at region δ_{H} 0.8 and 1.1 relatives to methyl groups identified two singlet hydrogen in δ_{H} 0.94 (CH_3 -C-18) and 0.63 (CH_3 -C-19) and four duplets in δ_{H} 0.82 (CH_3 -27), 0.85 (CH_3 -26); 0.91 (CH_3 -28), 1.03 (CH_3 -21).

^{13}C NMR spectra (Figure 2(b)) reveals C_{28} -sterol ergostane skeleton, including signals of six unsaturated carbons at δ_{C} 116.4- 141.5 corresponding to C-5 (δ_{C} 139.9); C-6 (δ_{C} 119.7), C-7 (δ_{C} 116.4), C-8 (δ_{C} 141.5), C22 (δ_{C} 135.7) and C23 (δ_{C} 132.1). Methyl carbons were observed in C-18 (δ_{C} 12.2), C-19 (δ_{C} 16.4), C-21 (δ_{C} 21.2), C-26 (δ_{C} 20.1), C-27 (δ_{C} 19.7) and C-28 (δ_{C} 17.7), whereas hydroxyl group was observed in C-3 (δ_{C} 70.6). All proton and carbon signals were assigned by

comparing the previously reported data (Alexandre *et al.*, 2017) (Table 4). All the evidence was identified as ergosterol (1), as shown in Figure 1.

Two isolated compounds from crude EtOAc extract were obtained from two pure compounds (2 and 3) as shown in Figure 1. Compound 2 was a yellow crystal, and the melting point was 175.7-176.9 °C. The FT-IR spectrum shows the peak of characteristic N-H stretching of the FT-IR spectrum at wavenumbers 3402 cm^{-1} and a C=O stretching of aldehyde at wavenumbers 1637 cm^{-1} . The peak at wavenumbers 3170 cm^{-1} corresponds to the =C-H stretching in aromatics.

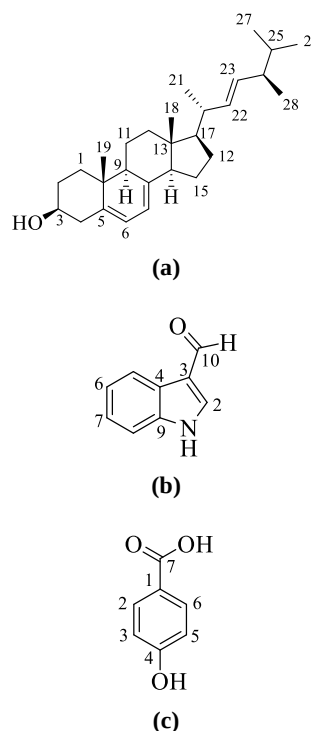
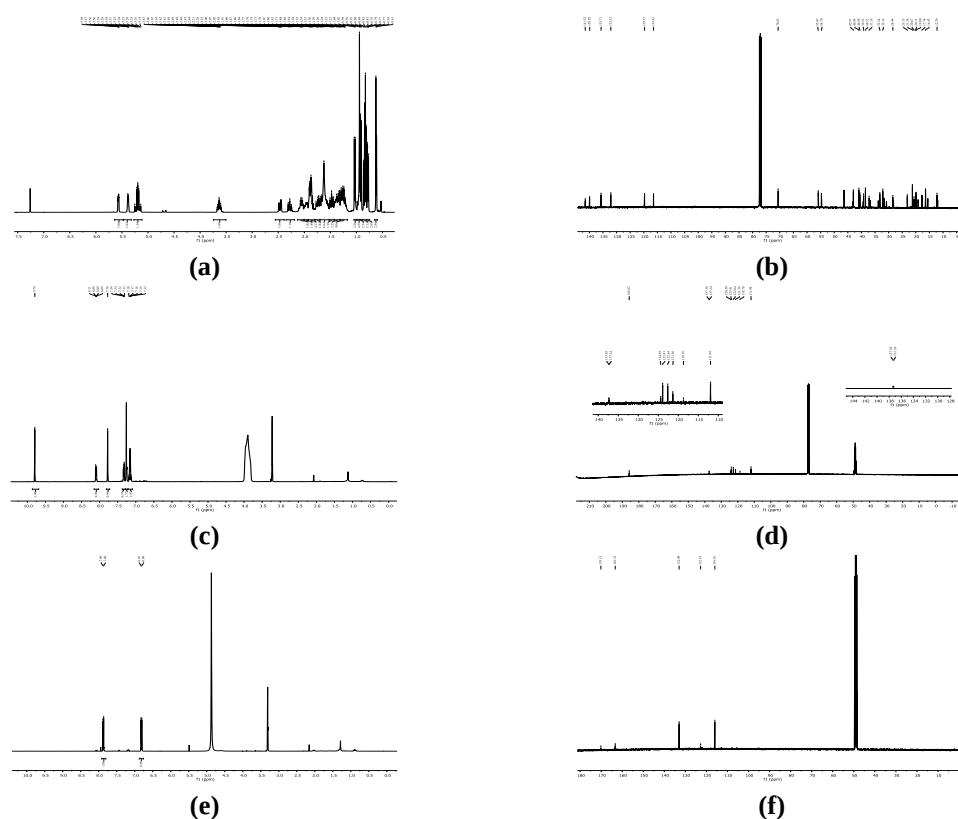


Figure 1. The isolated compounds (1-3) from crude hexane and EtOAc extract from *C. Comatus*.

The ^1H NMR spectrum (Figure 2(c)) showed signals for five methine protons (δ 7.17, 7.18, 7.78, 8.09, 8.11) and an aldehyde proton of CHO (δ 9.79).

The ^{13}C NMR spectrum (Figure 2(d)) presents eight olefinic carbons at C-9 (δ_{C} 111.94), C-3 (δ_{C} 118.70), C-6 (δ_{C} 121.36), C-7 (δ_{C} 122.64), C-8 (δ_{C} 123.91), C-5 (δ_{C} 124.39), C-10 (δ_{C} 137.24) and C-2 (δ_{C} 137.38). Furthermore, the C-4 (δ_{C} 185.82) revealed that the carbonyl of aldehyde functionality. These spectrometric data exactly coincide with those of indole-3-carbaldehyde (2) by comparing with previous reports (Nakajima *et al.*, 2002; Priya *et al.*, 2022) (Table 5).

Figure 2. ^1H and ^{13}C NMR spectrum of compound 1 (A-B), 2 (C-D) and 3 (E-F)Table 4. α -Glucosidase and Tyrosinase inhibitory activities of crude extract

Position	Compound 1				Position	Compound 1			
	$\delta_{\text{H}}^{\text{a exp.}}$	$\delta_{\text{H}}^{\text{lit}}$	$\delta_{\text{C}}^{\text{exp.}}$	$\delta_{\text{C}}^{\text{lit.}}$		$\delta_{\text{H}}^{\text{a exp.}}$	$\delta_{\text{H}}^{\text{lit.}}$	$\delta_{\text{C}}^{\text{exp.}}$	$\delta_{\text{C}}^{\text{lit.}}$
1			38.5	38.4	15			23.1	22.9
2			32.1	31.9	16			28.4	28.3
3	3.63 m (1H)	3.64 m (1H)	70.6	70.5	17			55.8	55.7
4			40.9	40.8	18	0.94 s (3H)	0.95 s (3H)	12.2	12.1
5			139.9	139.8	19	0.63 s (3H)	0.65 s (3H)	16.4	16.3
6	5.57 dd (5.5, 2.2 Hz, 1H)	5.58 dd (5.5, 3.0 Hz, 1H)	119.7	119.6	20			40.5	40.3
7	5.38 dd (5.4, 2.6 Hz, 1H)	5.38 dd (5.4, 2.9 Hz, 1H)	116.4	116.3	21	1.03 d ($J = 6.6$ Hz, 1H)	1.04 d ($J = 6.6$ Hz, 3H)	21.2	21.1
8			141.5	141.4	22	5.21 m (1H)	5.20 m (1H)	135.7	135.6
9			46.3	46.2	23	5.21 m (1H)	5.21 m (1H)	132.1	131.9
10			37.1	37.1	24			42.9	42.9
11			20.6	21.1	25			33.2	33.1
12			39.2	39.1	26	0.85 d ($J = 7.8$ Hz, 2H)	0.84 d ($J = 6.7$ Hz, 3H)	20.1	19.9
13			42.9	42.9	27	0.82 d ($J = 6.4$ Hz, 2H)	0.82 d ($J = 6.7$ Hz, 3H)	19.7	19.7
14			54.7	54.6	28	0.91 d ($J = 6.8$ Hz, 1H)	0.92 d ($J = 6.6$ Hz, 3H)	17.7	17.6

Table 5. ^{13}C and ^1H NMR data for compound 2 ($\text{CDCl}_3+\text{CD}_3\text{OD}$, 100 MHz and 400 MHz)

Position	Compound 2			
	$\delta_{\text{H}}^{\text{a}}$ exp.	δ_{H} lit. (recorded in 500 MHz in CD_3OD)	δ_{C} exp.	δ_{C} lit.
1	-	-	-	-
2	7.78 (1H, s)	8.17 (1H, s)	137.3	138.9
3	-	-	118.7	118.0
4	9.79 (1H, s)	9.92 (1H, s)	185.8	184.5
5	-	-	124.3	124.2
6	8.11 (1H, m)	8.20 (1H, d, $J=7.1$ Hz)	121.3	120.6
7	7.15 (1H, m)	7.28 (1H, dd, $J=8.3, 7.1$ Hz)	122.6	121.8
8	7.16 (1H, m)	7.33 (1H, dd, $J=8.3, 7.9$ Hz)	123.9	123.1
9	7.34 (1H, m)	7.52 (1H, d, $J=7.9$ Hz)	111.9	112.5
10	-	-	137.2	137.5

Table 6. ^{13}C and ^1H NMR data for compound 3 ($\text{CDCl}_3+\text{CD}_3\text{OD}$, 100 MHz and 400 MHz)

Position	Compound 3			
	$\delta_{\text{H}}^{\text{a}}$ exp.	δ_{H} lit.	δ_{C} exp.	δ_{C} lit.
1	-	-	122.0	127.5
2	7.87 (2H, d, $J=8.80$ Hz)	7.73 (2H, d, $J=7.95$ Hz)	132.9	131.1
3	6.81 (2H, d, $J=8.80$ Hz)	6.71 (2H, d, $J=7.95$ Hz)	116.0	114.4
4	-	-	163.3	159.9
5	6.81 (1H, d, $J=8.80$ Hz)	6.71 (2H, d, $J=7.95$ Hz)	116.0	114.4
6	7.87 (1H, d, $J=8.80$ Hz)	7.73 (2H, d, $J=7.95$ Hz)	132.9	131.1
7	-	-	170.1	169.4

Compound 3 was a white powder, and the FT-IR spectrum showed the O-H stretching at wavenumbers 3114 cm^{-1} . The C=O indicated the carbonyl of carboxylic acid at wavenumbers 1672 cm^{-1} . To confirm the structure of compound 3 was identified by the ^{13}C and ^1H NMR spectra.

The ^1H NMR spectrum (Figure 2(e)) showed one pair of doublet protons signal (δ_{H} 7.87 and 6.81, 2H, d, $J=8.80$ Hz), chemical equivalent, was found to have arisen from a para-substituted benzene ring.

The ^{13}C NMR spectrum (Figure 2(f)) presents 5 carbon signals but consists of 7 carbons because two of five show a higher than the other signal, which is mean to two carbons of a signal. The carbon signals are ascribable to the benzene ring carbons (δ_{C} 116.0 - 163.3) and carboxyl carbon (δ_{C} 170.1). Compound 3 was identified as 4-hydroxybenzoic acid based on spectroscopic data and comparison with the literature (Cho *et al.*, 1998). Shown in Table 6.

Molecular docking

The bioassay-guided of crude hexane and EtOAc extract isolations were shown to have α -glucosidase and tyrosinase inhibitory. The purification of both fractions was obtained from three pure major compounds, which are ergosterol (1), indole-3-carbaldehyde (2), and

4-hydroxybenzoic acid (3) from *C. Comatus*. Molecular docking analysis was studied to confirm of three major compounds were related to the strong inhibition of α -glucosidase and tyrosinase from crude hexane and EtOAc extract.

α -glucosidase interaction

The indole-3-carbaldehyde (2) is a small ligand and has one carbonyl and amine position. It can be inserted into the narrow channel of the active site and can form hydrogen bonds with ASP320 and ARG551, and also an electrical interaction with ASP461. The non-polar reaction was found only with TYR292. The binding interaction produced binding energies ranging from -5.29 to -5.58 kcal/mol. Post-docked molecules containing the highest binding energies (-5.58 kcal/mol) and lowest inhibition constant (81.62 μM) show in Figure 3(a-c). For 4-hydroxybenzoic acid (3) ligand, it has atoms that can be hydrogen bond donors and acceptors. Consequently, it raises the possibility of forming a hydrogen bond with amino acids. The docking result provide a specific position with binding free energy of -5.28 kcal/mol. As shown in Figure 3. (d), ASP198 is the main amino acid involved in interaction, providing both hydrogen bonds and electrostatic force. Ergosterol (1) is the largest. ligand that can bind to the active

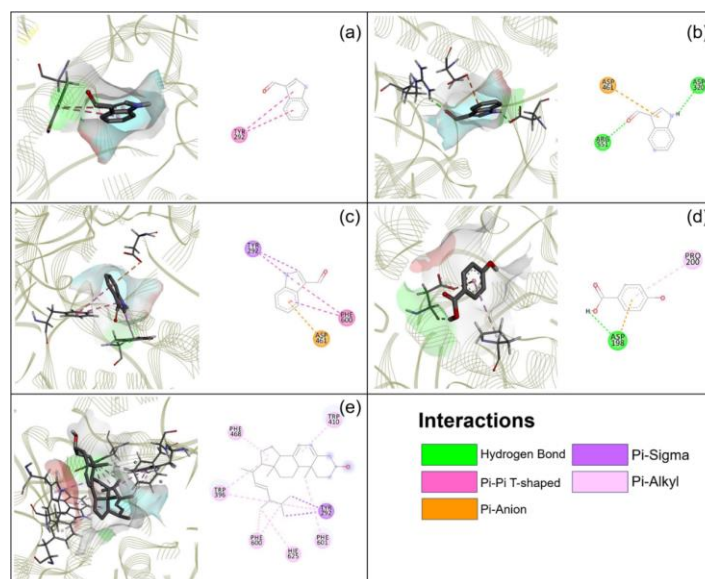


Figure 3. The different arrangement of ¹H-indole-3-carbaldehyde docked to α -glucosidase (a-c), the post docked structure of 4-hydroxy benzoic acid (d) and ergosterol (e) to α -glucosidase

Table 7. Binding free energy (ΔG), Inhibition constant (K_i), and detected receptor-ligand interaction of docked ligands to α -glucosidase

Receptor	Ligand	%Cluster site	Binding free energy, ΔG (Kcal/mol)	Inhibition Constant, K_i (μM)	Receptor-Ligand Interaction*
α -Glucosidase	indole-3-carbaldehyde	25.5	-5.58	81.62	TYR292(HDP)
		24.5	-5.50	92.57	ASP320(HB), ASP461(ELEC), ARG551(HB)
		32.5	-5.29	132.77	TYR292(HDP), ASP461(ELEC), PHE600(HDP)
	4-hydroxy benzoic acid	98.0	-5.28	134.78	ASP198(HB), ASP198(ELEC), PRO200(HDP)
	ergosterol	89.5	-9.91	54.18×10^{-3}	TYR292(HDP), TRP396(HDP), TRP410(HDP), PHE486(HDP), PHE600(HDP), PHE601(HDP), HIE625(HDP)

* HB is hydrogen bond, HDP is hydrophobic interaction and ELEC is electrostatic interaction.

site of α -glucosidase in a specific position and provides highest binding free energy (-9.91 kcal/mol). The high flexibility of the aliphatic portion of the ligand allows it to easily arrange itself into the surface's corners of the active site of the receptor. The majority of ligand-receptor interactions are non-polar, see Figure 2(e). Because the ligand has nonaromatic ring, its molecular structure is not very rigid, and the alicyclic portion is flexible. As a result, it can adapt to the amino acid surface of various amino acids (Table 7) in the active site to contribute to better interactions. According to the findings, ergosterol (1) and 4-hydroxybenzoic acid (3) are promising candidate structures that can be further developed as drugs to inhibit α -glucosidase activity because it is quite

specific to this enzyme. Especially for ergosterol (1), adding a variety of functional groups to a molecular structure may increase the number of interactions.

Tyrosinase interaction

Three ligands (ergosterol (1), indole-3-carbaldehyde (2) and 4-hydroxybenzoic acid (3)) were docked to tyrosinase. Considering the %cluster sites, Table. 8, all three ligands were found to have no significant tyrosinase specificity. Although only 21-28% of the cluster positions are occupied, Ergosterol (1) has the strongest binding interaction among all ligands. This strong binding energy (-9.64 kcal/mol) is because of the large molecular size and the abundance of hydroxyl groups.

Table 8. Binding free energy (ΔG), Inhibition constant (K_i), and detected receptor-ligand interaction of docked ligands to Tyrosinase

Receptor	Ligand	%Cluster site	Binding free energy, ΔG (Kcal/mol)	Inhibition Constant, K_i (μM)	Receptor-Ligand Interaction*
Tyrosinase	indole-3-carbaldehyde	24	-6.22	27.58	ASN260(HB), MET280(HB)
		23	-5.43	104.07	ASN260(HB), GLY281(HB), VAL283(HB), VAL283(HDP)
	4-hydroxy benzoic acid	25	-5.14	169.94	HIS259(HB), ASN260(HB), MET280(HB), SER282(HDP), VAL283(HDP), ALA286(HDP)
		28	-5.00	217.73	HIS263(HB), HIS263(HDP), VAL283(HDP), ALA286(HDP)
	ergosterol	21.5	-9.64	86.2×10^{-3}	HIS61(HDP), HIS85(HDP), HIS244(HDP), VAL248(HDP), HIS259(HDP), HIS263(HDP), PHE264(HDP), VAL283(HDP), HIS296(HDP)

* HB is hydrogen bond, HDP is hydrophobic interaction and ELEC is electrostatic interaction.

Conclusion

The crude hexane and EtOAc extract from *C. Comatus* showed the highest inhibition of α -glucosidase and tyrosinase activities inhibitory, which is better than their commercially available inhibitors. This bioassay-guided isolation revealed that, ergosterol (1) was isolated from crude hexane fraction, while two compounds, indole-3-carbaldehyde (2), and 4-hydroxybenzoic acid (3), were isolated from the EtOAc extract fraction. The molecular docking of compounds 1-3 were docked to α -glucosidase and tyrosinase. Interestingly, ergosterol (1) and 4-hydroxybenzoic acid (3) reveal their specific binding to α -glucosidase enzyme, which provides their highest binding free energy at -9.91 and -5.28 kcal/mol, respectively. This evidence was in accordance with their highest anti- α -glucosidase activity of crude hexane and EtOAc extract fractions. Therefore, *C. Comatus* mushroom is applicable for the functional food, which contains rich compound to inhibit α -glucosidase. It is of great interesting for the development of new functional food products. Furthermore, *C. Comatus* mushroom is applicable as cosmetic additives due to crude hexane and EtOAc extract fractions inhibiting tyrosinase enzyme. The clinical effectiveness of these crude extracts should be further investigated in future research, which develops new resources for treating diabetes mellitus and cosmetic additives.

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