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ISOLATION OF α-MANGOSTIN FROM MANGOSTEEN (*Garcinia mangostana* L.) PEELS AND EVALUATION OF ITS INHIBITORY ACTIVITY TOWARD α-GLUCOSIDASE AND α-AMYLASE IN THE COMBINATION WITH ACARBOSE

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SUMMARY

Using the natural agents with inhibitory activity against digestive enzymes α-glucosidase and α-amylase capable of hydrolyzing carbohydrates into glucose to reduce blood glucose levels in the blood is one of the effective strategies to control diabetes, especially type II diabetes. α-Mangostin (AMG) was proven to have strong biological activities, such as antifungal, antibacterial, anti-inflammatory, anti-cancer. However, the evaluation of the antidiabetic activity of this substance through inhibition of starch hydrolytic enzymes activity has not been fully carried out, especially when they are combined with commercial drugs, such as acarbose. In this study, AMG was isolated from the peels of the mangosteen grown in Vietnam using a simple isolation process with two steps: i) fractionation of the material in n-hexane solvent, and ii) chromatography of n-hexane fraction on a silica gel column combined with crystallization. The α-glucosidase inhibitory activity (AGI) and α-amylase inhibitory activity (AAI) of purified AMG alone or in combination with acarbose were then determined spectrophotometrically. The obtained results indicated that AMG had a purity of > 98% by HPLC examination and its chemical structure was confirmed by NMR spectra analysis combined with the reference. The isolated AMG showed good AGI and AAI with IC_{50} values of 8.25 µg/mL and 24.5 µg/mL, respectively. The AGI increased to 69.4% when AMG (5.0 µg/mL) was combined with acarbose at a concentration of 2.5 µg/mL, while the AAI did not have a clear synergistic effect. Our finding suggests the possibility of using the combination formula to enhance acarbose efficacy in treatment of the disease.

Keywords: α-mangostin, α-amylase, α-glucosidase, mangosteen (Garcinia mangostana L.), diabetes

INTRODUCTION

Diabetes mellitus, also known as diabetes, is a group of disorders in the metabolism of carbohydrates, fats, and proteins when the insulin of the pancreas is deficient or reduced in its action in the body, manifested by consistently high blood sugar levels. Diabetes is one of the main causes of many serious diseases, typically coronary heart disease, cerebrovascular accident, blindness, kidney failure, which, if not treated promptly, can lead to death (Nguyen Huy Cuong, 2010).

According to the World Health Organization (WHO), more than 346 million people worldwide have diabetes. This number is likely to double by 2030 with nearly 80% of diabetes deaths occurring in low- and middle-income countries (*https://www.who.int/news-room/fact-sheets/detail/diabetes*). The International Diabetes Federation (IDF) reported that Vietnam has about 3.53 million people living with diabetes, of which 70% of people with diabetes have not been diagnosed (data in 2017).

Currently, there are a series of drugs launched to treat and limit the development of diabetes patients effectively, such as insulin, biguanide... However, the use of synthetic agents often causes side effects and high costs. Herbal products were recommended by WHO as a safe and effective alternative (Ekor, 2013). The herb is an available source of raw materials, low cost, and shows fewer unwanted effects to the body. Vietnam has a rich and diverse source of herbs, which have been traditionally applied to treat diabetes, such as pandan leaves *Ananas comosus*, garlic (*Allium sativum*), guava leaves (*Psidium guajava*), bitter squash (*Momordica charantia* L.) (Do Tat Loi, 2006; Le Ngoc Thanh, Nguyen Thi Bay, 2009). Therefore, the development of blood-sugar-lowering drugs derived from medicinal herbs for the treatment of diabetes has attracted much attention.

One of the effective ways to control blood sugar after a meal is to inhibit the activity of the digestive enzymes, including α-glucosidase (AGO) and α-amylase (AA), which can hydrolyze carbohydrates into glucose. The enzyme inhibitory activity slows the absorption of carbohydrates in the small intestine, thereby reducing blood glucose levels. This is one of the targets used effectively to control diabetes, especially type 2 diabetes (Hau *et al.*, 2018; Chiba, 1997; Huyen *et al.*, 2013).

Mangosteen *(Garcinia mangostana* L*)* belongs to the family *Clusiaceae*. This is a common tropical plant in Southeast Asia, including Vietnam. This plant is very rich in xanthone derivatives, especially AMG which was proved to exhibit strong biological activities, such as antifungal, antibacterial, anti-inflammatory, anti-cancer (Ibrahim et al., 2016)… Although the evaluation of antidiabetic activity through inhibition of starch hydrolytic enzymes activity of this substance has been investigated, the examination of its AGI and AAI in combination with a commercial drug, such as acarbose has not been investigated. This study presents the results of AMG isolation using a new isolation process and evaluation of the AGI and AAI of the purified AMG alone or in combination with the antidiabetic drug acarbose as a basis for treatment application.

MATERIALS AND METHODS

Plant material

Mangosteen peels (*Garcinia mangostana* L.) were purchased from the markets in Hanoi. The material was cut into small pieces, dried in an oven at 50^{o}C, and then ground into a fine powder.

Methods

Preparation of fractional extract

Mangosteen peel powder (1000 g) was soaked in 96% ethanol (EtOH) for 3 days. The extraction process was repeated 3 times. The ethanol extract was obtained by vacuum drying at < 50^{o}C. The ethanol extract was then mixed with water and fractionated twice with n-hexane solvent at a ratio of 1:1 by volume. The n-hexane layer was collected and dried using a rotary vacuum distillation machine at 50 atm and 50^{o}C to obtain the residue (8.6 g) for further isolation steps.

Isolation of AMG from the mangosteen peels

In this experiment, the n-hexane fraction, which is rich in AMG was loaded on a column packed with silica gel beads of size 60-230 mesh (Merck) using hexane:acetone (3:1) solvent system. The selected fractions were then pooled, crystallized, and checked for purity by thin-layer chromatography (TLC) (silica gel 60 F254 sheets, Merck) and high-performance liquid chromatography (HPLC). The chemical structure of AMG was confirmed by analysis of

nuclear magnetic resonance (NMR) spectra compared to the references.

Determination of alpha-glucosidase inhibitory activity (AGI)

AGI was determined by the quantitative spectrophotometrically method (Tadera *et al.*, 2006). Briefly, on a 96-well plate, add to each well the AGO enzyme with a final concentration of 0.05 U/mL prepared in 20 mM phosphate buffer pH 6.8. Then, the test agent was added and incubated with the enzyme for 5 min. Next, pNPG was added at a concentration of 600 μM. The reaction mixture was further incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.2 M Na_2CO_3 solution. Then, the plate was spectrophotometrically measured at 405 nm. The inhibitory activity of the test sample was determined and the concentration of 50% inhibitory enzyme activity (IC_{50}) was calculated. The positive control was acarbose (Sigma).

Determination of alpha-amylase inhibitory activity (AAI)

AAI was measured using a microplate-based starch–iodine assay (Pongphasuk *et al.*, 2005; Xiao *et al.*, 2006). Assay reaction was initiated by adding 40 μL of starch (Sigma S-2630) solution (2.0 g/L) and 40 μL of enzyme in 0.1 M phosphate buffer at pH 7.0 to microplate wells. The enzyme was incubated with the test agent for 5 minutes before adding the substrate (soluble starch) which was prepared in water. To minimize evaporative loss during incubation, a plastic mat was used to cover the microplate. After 30 min of incubation at 37 °C, 20 μL of 1M HCl was added to stop the enzymatic reaction, followed by the addition of 100 μL of iodine reagent (5mM I_2 and 5mM KI). Following color development, the absorbance at 580 nm was measured using a microplate reader (Bio-TEK EL x 808 microplate reader, USA).

Determination of cytotoxicity of AMG

Mouse fibroblast cells NIH3T3 was used to determine the cytotoxicity of the isolated compounds and fractions. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/mL penicillin, and 100 ug/mL streptomycin and incubated at 37ºC in 5% CO_2. The tested samples were dissolved in DMSO at different concentrations, added into the culture medium, and incubated for 24 h. The cells viability was determined using an MTT assay in which 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced to formazan (Scudiero *et al*., 1988). MTT (5 mg/mL in PBS) was added to each well and incubated for 4 h. The formazan crystal was dissolved in DMSO. The absorbance values of the solutions were measured at 570 nm using a plate reader (BioTek ELx808 microplate reader, USA). The IC_{50} values of the test samples were analyzed and compared to evaluate cytotoxicity.

Data analysis

The data were processed by statistical methods using standard t-test or ANOVA in the case of comparing multiple samples with $p < 0.05$.

RESULTS AND DISCUSSION

Isolation of α-mangostin from the peels of mangosteen

The n-hexane fraction was loaded on a silica gel chromatographic column (3 x 80 cm) and eluted with a solvent system of n-hexane:acetone = 3:1 (v/ v). The purity of fractions (10 mL) was determined by thin-layer chromatography (TLC). The results showed that there were no substances in fractions 1-4. Fractions of 5-10 contained the group of substances with higher R_f. Fractions of 11-31 contained both high and low R_f substances. Fractions of 33-63 contained only one band with low R_f. Fractions of 65-73 appeared with 2 bands. The results (Figure 1) showed that fractions 35-64 contained only one band equivalent to standard AMG (M) in TLC, suggesting the interest substance was purified.

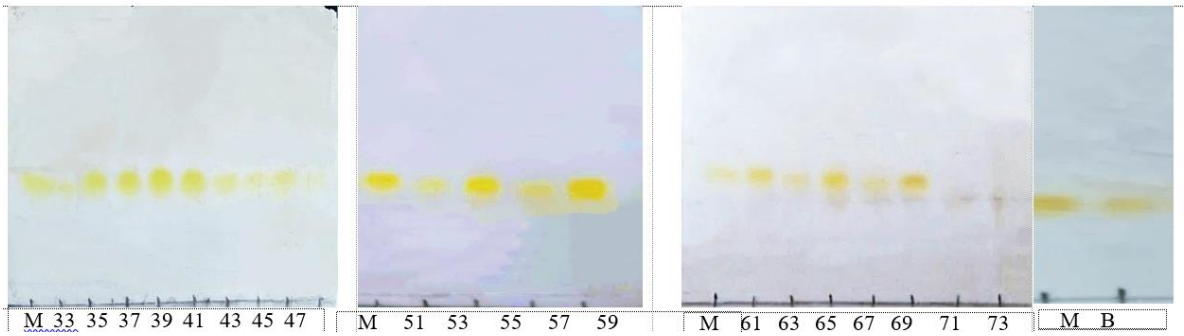

Figure 1. Chromatography of n-hexane fraction on silica gel column using the eluting solvent system of n-hexane:acetone = 3:1 (v/v). M. Standard AMG; B. Isolated compound**.**

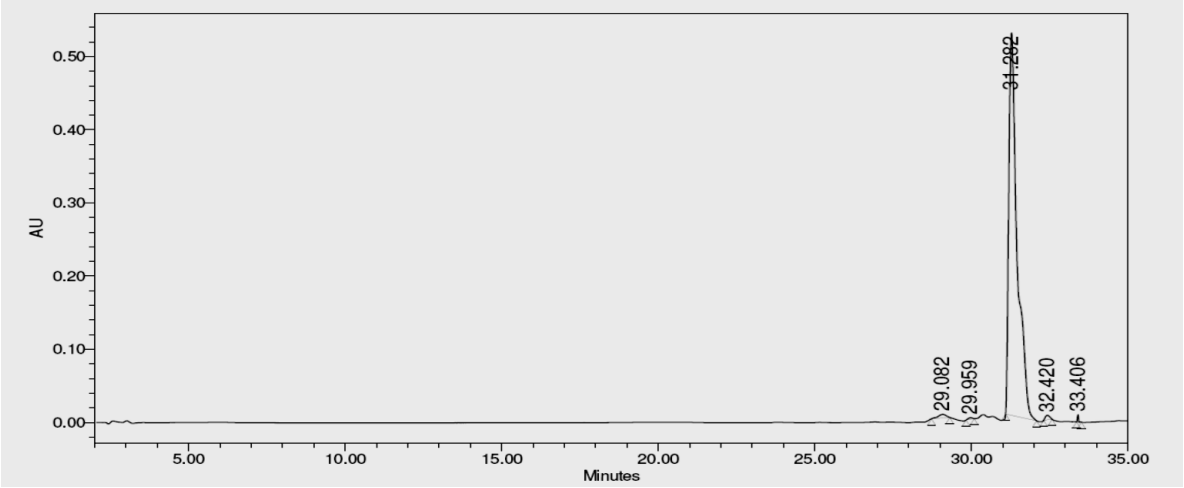

Figure 2. HPLC chromatogram of the isolated compound (Hitachi – DAD L2455).

Table 1. AMG purity compared to the standard AMG measured by HPLC.

	RT	Area	% Area	Height
1	29.082	85349	0.87	4957
2	29.959	19971	0.20	2180
3	31.282	9657452	98.10	522807
4	32.420	59313	0.60	6209
5	33.406	22526	0.23	9867

The selected fractions of 5 mL were pooled and crystallized at room temperature to yield the pure compound. HPLC combined with the DAD detector of the isolated compound showed a major peak with a retention time of 31.282 minutes and a purity of > 98%, equivalent to that of a standard AMG (Table 1, Figure 2). Analysis of the NMR data (Table 2) and comparison with reference data (Kaomongkolgit *et al.*, 2009) confirmed that the purified substance was AMG with the chemical formula of $C_{24}H_{26}O_6$ and the molecular weight is 410 (Figure 3).

Thus, using silica gel chromatography with a solvent system of n-hexane:acetone (3:1), combined with crystallization, AMG was successfully isolated from the n-hexane fraction with a purity of > 98%, and an isolation yield of 0.13%. This procedure is simple and more efficient than those of previously reported processes (the yield of 0.13% compared to 0.01% and 0.1% with the same high purity of > 98% (Nguyen Thi Mai Phuong, Marquis, 2011; Do Thi Tuyen *et al.*, 2010).

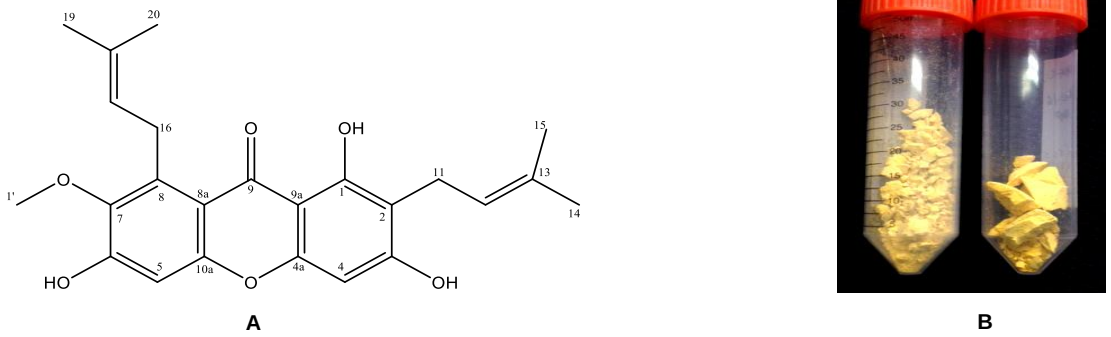

Figure 3. Chemical structure of AMG ($C_{24}H_{26}O_6$). A. Chemical structure; B. Isolated AMG.

Table 2. Analysis data of ^{1}H và ^{13}C spectra of isolated AMG

Pos	$\delta_C^{a,c}$	$\delta_H^{a,d}$ (mult., *J* = Hz)
1	159.8	-
2	109.9	-
3	162.2	-
4	92.2	6.34 (s, 1H)
4a	154.1	-
5	101.8	6.80 (s, 1H)
6	156.8	-
7	143.3	-
8	136.3	-
8a	109.6	-
9	181.2	-
9a	109.7	-
10a	154.5	-
11	20.9	3.22, 3.20 (d, 2H, *J* = 3.5Hz)
12	123.7	5.17 (m, 1H)
13	130.3	-
14	25.4	1.62 (s, 3H)
15	17.9	1.62 (s, 3H)
16	25.5	4.02, 4.01 (d, 2H, *J* = 5.0Hz)
17	122.5	5.15 (m, 1H)

18	130.2	-
19	17.6	1.73 (s, 3H)
20	25.7	1.77 (s, 3H)
1'	*60.1*	*3.70 (s, 3H)*

[a]Measured in DMSO, [c]125 MHz, [d]500 MHz.

AGI and AAI activities of AMG

Controlling the absorption of glucose produced from the breakdown of starch hydrolysis by inhibiting the pancreatic AA and by limiting the absorption of glucose by inhibition of intestinal AGO are the two available therapeutic approaches for type II diabetes management. In our study, AMG exhibited good activities in a dose-dependent manner. The 50% of inhibition (IC_{50}) values were 8.25 ug/mL (for AGI) and 25.4 ug/mL (for AAI) while those of acarbose were 4.82 µg/mL and 19.6 µg/mL, respectively, Figure 4).

In addition, we evaluated the AGI and AAI of AMG in combination with commercial antidiabetic drug acarbose at a selected concentration. The representative combination formula here was selected based on a data series of tested concentrations for each compound in combination to have a highest synergistic inhibitory effect (data not shown).

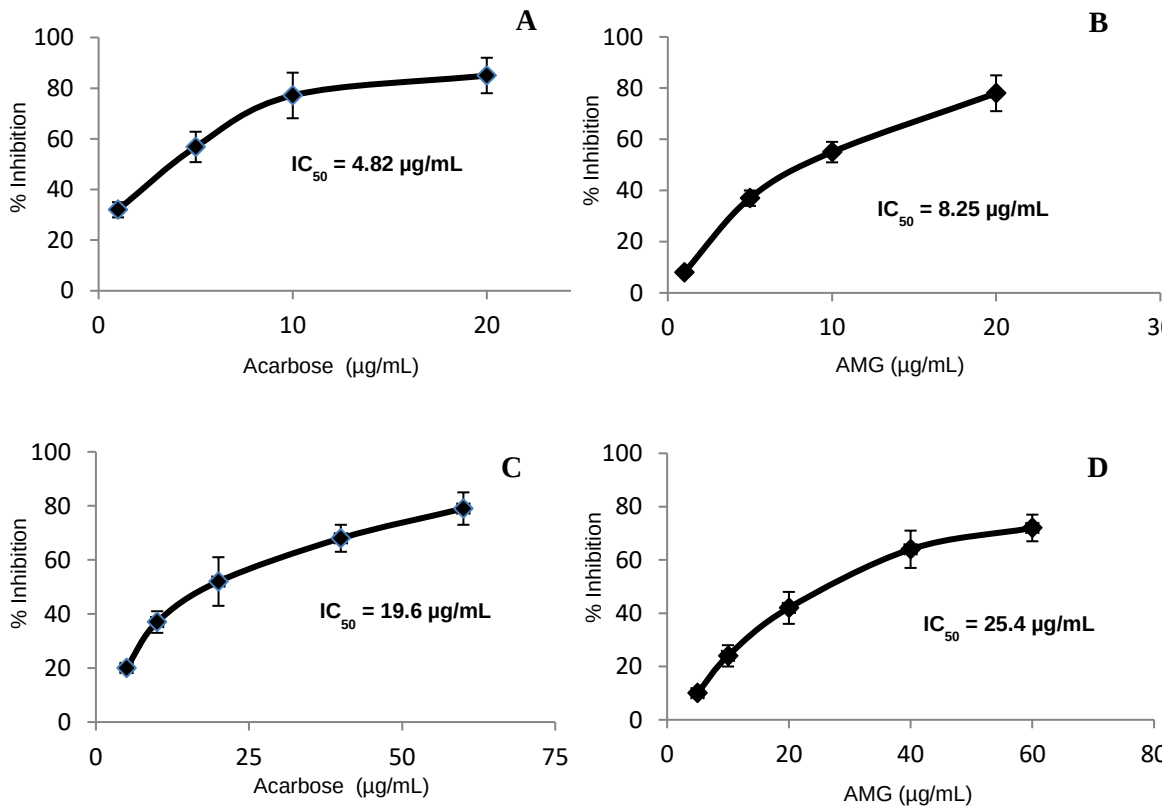

Figure 4. Effect of AMG on activity of AGO (A, B) and AA (C, D).

Table 3. Synergistic AGI activitiy of AMG in combination with acarbose.

AMG (µg/mL)	Acarbose (µg/mL)	AGI (% Inhibition)
5	0	51.5 ± 3.2
0	2.5	28.5 ± 1.3
5	2.5	69.4 ± 2.9(*)

Data are expressed as the mean ± SD. The value marked with an asterisk(*) is significantly different as compared with AMG and acarbose single groups (pair-wise comparison using Student's t test with $p < 0.05$).

The data in Table 3 showed that in the single forms, AMG at the concentration of 5 µg/mL had the AGI value of 51.5% and acarbose had the AGI value of 28.5% at the concentration of 2.5 µg/mL. In presence of 5 µg/mL AMG in combination with acarbose at 2.5 µg/mL, its inhibition rate was enhanced to 69.4% ($p < 0.05$). Meanwhile, the AAI activity seems not to be enhanced in presence of this combination and other combinations at higher concentrations (data not shown). Clearly, further works need to be carried out to understand the reason for this difference. Thus, the result here showed a synergistic effect on AGI activity of the AMG – acarbose combination formula of AMG (5 µg/mL) and acarbose (2.5 µg/mL), suggesting the possibility of using the formula for the disease treatment.

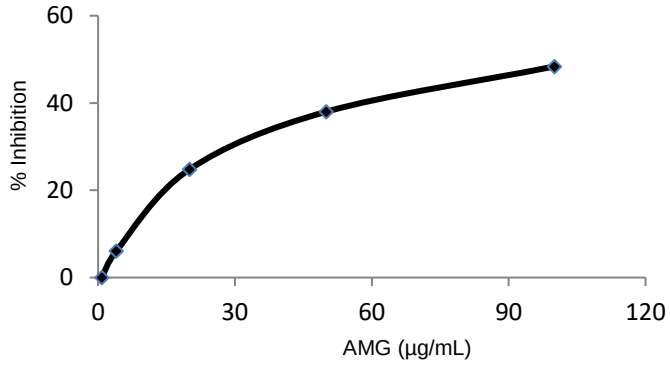

Figure 5. Cytotoxicity on NIH 3T3 mouse fibroblast cells of AMG.

Evaluation of cytotoxicity of AMG

Data on cytotoxicity and acute toxicity of xanthones in mangosteen peel extract in general or AMG, in particular, have been mentioned in several recent studies (Sornprasit *et al.*, 1987) demonstrated that mangosteen peel extract at doses of 1.0, 2.0 and 3.0 g/kg body weight of rats did not cause toxicity nor change parameters hematology or visceral weight of rats (Pongphasuk *et al.*, 2005) also did not find that the mangosteen peel extract had a lethal effect. The AMG toxicity test doses of up to 4,000 µg/mL for 480 min also showed no cytotoxic effects on human gingival fibroblasts (Kaomongkolgit *et al.*, 2009). AMG had low toxicity on normal human epithelial ovarian cells SV40 with an IC_{50} value of 93.26 ± 3.92 µM after 24 h treatment compared to 24.5 µM on Hela cancer cells (Habbash *et al.*, 2017). Novilla *et al.*, (2016) also indicated that AMG had less effect on the normal lymphocyte cells from two different donors compared to the cancer cells. The acute toxicity of AMG was also evaluated by (Samankaranarayan *et al.*,1979) and (Sornprasit *et al.*, 1987) in rats. The authors found that AMG administered orally to rats at a dose of 200 mg/kg body weight/day for 6 days had no adverse

effects on the rats. AMG had no reported toxicological evaluations *in vivo* (Ibrahim et al., 2015). In our experiment, the cytotoxicity of AMG on the mouse fibroblast cells was examined. Our obtained data in this experiment indicated that the IC_{50} value of AMG was > 100 µg/mL (Figure 5), much higher than the concentrations showing AGI and AAI activities.

CONCLUSION

AMG from the mangosteen peels has been successfully isolated using a simple purification procedure consisting of two steps: i) fractionation extraction with n-hexane; ii) chromatography of n-hexane fraction on a silica gel column using an eluting solvent system of n-hexane:acetone (3:1, v/v) combined with crystallization. The isolated AMG has a purity of > 98%. AMG exhibited good AGI and AAI with IC_{50} values of 8.25 µg/mL and 25.4 µg/mL, respectively. The AGI activity increased from 51.5% to 69.4% when AMG (5 µg/mL) was combined with acarbose (2.5 µg/mL). This is a new finding, suggesting the applicability of the combination formula in the treatment of diabetes. More detailed studies on its synergistic effects and toxicity *in vivo* are needed to confirm its therapeutic applications.

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