Original Article

α- Glucosidase Inhibitory and Antioxidant Activities of *Moluccella aucheri* (Boiss.) Scheen Extracts

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Abstract

Background & Objective: α-Glucosidase is one of the main enzymes in the intestinal absorption of carbohydrates. Inhibition of this enzyme can improve postprandial hyperglycemia in diabetic patients. Also, antioxidants can ameliorate diabetes complications resulted from oxidative stress. In this study α-glucosidase inhibition, antioxidant activity and total phenol content of different extracts and obtained fractions of *Moluccella aucheri* have been evaluated.
Materials & Methods: The ethanol extract of aerial parts of *M. aucheri* was fractionated using liquid extraction method with petroleum ether, dichloromethane and ethyl acetate, respectively. The extracts and the resulting fractions of *M. aucheri* were tested against α-glucosidase enzyme from yeast using an in vitro colorimetric model at λ 405 nm. Antioxidant activity was assessed using two different methods, ferric reducing antioxidant power (FRAP) and the scavenging activity of DPPH radicals' methods.
Results: The screening results indicated that the antioxidant activity and total phenolic content of the methanol extract were potentially higher than ethanol extract, while the ethanol extract inhibited α-glucosidase enzyme to a greater extent. Ethyl acetate fraction illustrated potentially the highest antioxidant activity and phenolic content, but its inhibition activity against α-glucosidase was placed after the petroleum ether fraction. Two previously isolated methoxy flavones; genkwanin, 5-hydroxyl-7, 4'-dimethoxyflavone inhibited the α-glucosidase enzyme strongly.
Conclusion: Since the ethyl acetate extract has both considerable antioxidant activity and α-glucosidase inhibitory activity, it may be considered as a potential crude drug for diabetes.

Keywords: diabetes, *Moluccella aucheri*, α-glucosidase, antioxidant

Introduction

Diabetes is one of the most important metabolic diseases that leads to hyperglycemia and is the result of the lack of insulin or its function (1). In 2019, approximately 463 million adults between the ages of 20-79 were diagnosed with diabetes that caused the death of 4.2 million people worldwide. It is estimated that diabetic patients rise by 700 million people by 2045 (2). Therefore, the treatment of diabetes is considered one of the most important research topics.

One of the ways which control the blood sugar levels is using α-glucosidase inhibitors that prevent cleavage of oligo- and polysaccharides to absorbable sugar, D-glucose (3). Different phytochemicals especially flavonoids, phenolic

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acids, tannins and anthocyanin may act as antidiabetic nutraceuticals and as antidiabetic agents by inhibiting both α-glucosidases and α-amylases (4-9). In addition to enzyme inhibitory effects, the phenolics are known to act as powerful radical scavengers and antioxidants (10, 11). Therefore, these phytochemicals may have dual actions of antioxidant-antidiabetic and be useful in the treatment or prevention of complications of diabetes by preventing oxidative stress. Oxidative stress occurs by an imbalance between oxidative species (ROS and RNS) and antioxidants (12) lead to vascular complications of diabetes such as cardiovascular disease, nephropathy, retinopathy, neuropathy (13). Oxidative stress (OS) management offers a new therapeutic strategy to prevent diabetes complications.

Moluccella aucheri (Boiss.) Scheen (syn. *Otostegia aucheri* Boiss.) belongs to family Lamiaceae and is one of the medicinal plants that have antioxidant and antidiabetic activities (14, 15). *M. aucheri* is a perennial subshrub, growing 30 to 60 cm; with leaves of a narrow entire, pale green, spinose-apiculate (pungent) and a glabrous; flowering branches, multiple flower cycles; calyx of funnel- shaped; corolla 2-lipped, white, lower three-lobed and small white flowers (16). The methanolic extract of an accession from Pakistani species of the plant exhibited hypoglycemic effect in type 2 diabetes in the animal model test although its action mechanism has not been determined yet (17). The *Moluccella* genus encompasses eight species which are native to Asia and the Mediterranean (18) while *M. aucheri* is distributed in only Southern parts of Iran and Pakistan (19) and is used as a strengthener of hair and gums in folk medicine (20). There are a few *phytochemical* and biological activity reports on this species in the literature. Recently, we have isolated one labdane diterpenoid, two phytosterols and two flavonoids; genkwanin (1), 5-hydroxyl-7, 4′-dimethoxyflavone (2, Figure 1.) from a dichloromethane extract of M. aucheri (21).

In the present study, α-glucosidase inhibitory, antioxidant activities and total phenol contents of different extracts and resulting fractions of M. aucheri have been reported for the first time.

Material and methods

Reagents and chemicals

α-glucosidase (Sigma, Germany), silica gel (70–230 mesh) for column chromatography, TLC aluminum sheets, reagents of 2, 4, 6-tripyridyl-s-triazine (TPTZ), ferric chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), Folin-Ciocalteu and 2, 2-diphenyl-1-picrylhy-drazyl (DPPH) were obtained from Merck (Darmstadt, Germany).

Plant material

The aerial parts of *Moluccella aucheri* (Boiss.) Scheen were collected in May 2017 from Hormozgan province in Southern Iran which is located in the geographical range of east longitude 57° 12′ 29″- 57° 11′ 25″ and north latitude 27° 46′ 6″ – 27° 44′ 21″. The plant was identified by Mr. Mehdi Zare, the plant taxonomist and a voucher specimen (PC-96-3-23-3.1) was deposited in the Herbarium of Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences (MNCRC), Shiraz, Iran.

Extraction and fractionation of the plant material

The aerial parts of *M. aucheri* (500 g) were extracted by maceration in 96% ethanol (EtOH; 5 L× 3). After drying the extract in rotary evaporator in reduced pressure and at 40 °C, different fractions were prepared using liquid-liquid extraction (LLE). The ethanol extract (6.3 gr) was dissolved in a mixture of methanol and water (200 mL, 50/50 v/v) in a separating funnel, then extracted with petroleum ether and after removal of the methanol of the aqueous phase, with dichloromethane (DCM) and ethyl acetate (EtOAc) solvents (each 3 × 200 ml), successively. The petroleum ether (1090 mg) and DCM (2102 mg) and EtOAc fractions (692.4 mg) were obtained as concentrated precipitates or gummy material upon removal of their solvents in the reduced pressure at 40 ˚C. To prepare the crude methanol extract, the fresh aerial parts of the plant (100 g) were macerated in methanol (MeOH) solvent (1 L ×72h) and finally the crude extract of MeOH (yield 1.7%) was prepared upon evaporation of its solvent at the above-mentioned condition.

α-Glucosidase enzyme inhibition assay

The inhibitory effects of the samples on α-glucosidase (from yeast EC 3.2.1.20) activity was evaluated by in vitro model (22) with some modifications. In vitro assay was performed using p-nitrophenyl-D-glucopyranoside (PNPG) as a substrate in a colorimetric reaction that is hydrolyzed by the enzyme to glucose and p-nitrophenol (yellow). The activity of the enzyme is determined by measuring the adsorption of p-nitrophenol at λ 450 nm, produced in the solution. Briefly, 5 µL of the samples in DMSO at five final concentrations (0.5-2.0 mg/mL) were added to a mixture of 20 µL α-glucosidase (0.5 U/mL) and 115 µL phosphate buffer (pH= 6.8, 100 mM). After incubating at 37 ˚C for 10 min, the reaction started by addition of 20 µL of 2.5 mM PNPG and was incubated at 37 ˚C for further 30 minutes. The reaction was terminated by adding 80 µL of Na_2CO_3 (100 mM). The absorbance was read at λ 405 nm using a microplate reader. The DMSO was used instead of samples in control groups while acarbose was used as a positive control. To correct the background absorbance, the enzymes were replaced by the buffer the in the blanks. The percentage of inhibition was calculated and IC_{50} value was determined using Curve Expert software (version 1.3).

% Inhibition= ((Abs (Control)-Abs (Sample)))/(Abs (Control)) × 100

Antioxidant activity and total phenol contents

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Antioxidant activity was assessed by the scavenging activity of DPPH radical methods (23). Five µL of samples solutions at five different concentrations were added to 195 µL DPPH solution (0.1 mM) in 96-well plate to afford the final concentrations of 15.6- 250 µg/mL. Then microplate was shaken for 30 minutes in the dark at room temperature. The absorption of the solution was read by a microplate reader at λ 517 nm against the blank. The reaction mixture without sample was considered as the bank. After calculating the percentage of radical scavenging activity, half maximal inhibitory concentration (IC_{50}) values were calculated with Curve Expert software (version 1.4). Quercetin was considered as positive control.

Radical scavenging activity % = (Ab control – Ab sample)/(Ab Control) ×100

Ferric reducing antioxidant power (FRAP) antioxidant assay

In Ferric Reducing Antioxidant Power (FRAP) assay (24), antioxidants act as reducing agents in the redox reaction and reduce Fe3+ to Fe^{2+}.At first, the FRAP reagent was prepared in acetate buffer (0.03 M) in pH=3.6, $FeCl_3$ (0.02 M), 2, 4, 6-tripyridyl-s-triazine (TPTZ) (0.01 M) dissolved in HCl (0.04 M) with ratio (10:1:1). Then 20 µL of samples at five final concentrations 250-15.6 µg/mL were added to 180 µL of FRAP reagent. The microplate was incubated for 30 min at room temperature and the absorbance was read at λ 593 nm. The standard calibration curve of $FeSO_4$.7H2O was plotted. Results were expressed as mg $FeSO_4$ equivalent to g of extract.

Total phenolic contents (TPC) test

The total phenol contents of the extracts were evaluated by Folin-Ciocalteu reagent (23). Briefly, 5 µL of the sample solution, 145 µL distilled water and 20 µL Folin-Ciocalteu reagents were mixed by vortex. The above solutions were incubated 8.5 min at room temperature, then 30 µL of a 0.25% sodium carbonate solution was added. The above reaction mixtures were kept in the dark at room temperature for 2 h followed by measurement of their absorbance at λ 765 nm against the blank. The concentrations of the total phenolic in the plant extracts were calculated compared to a series of gallic acid standard solutions and expressed as mg equivalent of gallic acid in 1 g extract.

Statistical analysis

Data analysis was performed by SPSS software (version 19.0) using one-way ANOVA followed by the Tukey post hoc test. P value < 0.05 was considered statistically significant. Pearson's correlation coefficients of data were done using the SPSS software.

Results and discussion

The antioxidant (DPPH and FRAP), total phenol content (TPC) and α-glucosidase inhibitory activity were measured for different extracts and fractions of M. aucheri (Table 1).

Since the ethanol extract inhibited the enzyme better ($P < 0.05$) than that of methanol while its antioxidant activity and TPC were comparable to that of methanol we chose the earlier extract for further fractionation and performing the assays on them. Among the tested fractions and extracts, the ethyl acetate showed the highest TPC and the most powerful antioxidant activity including the lowest DPPH, $IC_{50} = 66.6 \pm 1.5$ µg/mL and highest FRAP values, 367.4 ± 2.7 mg $FeSO_4$ equivalent of g extract (EGE), respectively. Although the petroleum ether fraction was the most active enzyme inhibitor ($IC_{50} = 217.0 \pm 5.5$ µg/mL), due to the less water solubility and the lack of antioxidant activity it may be the second choice for further drug development investigations. This fraction exhibited an inhibition rate of over 80% at all concentrations above 0.5 mg/mL (Chart 1).

Many studies reported the inhibitory activity of different plant extracts against α-glucosidase. The inhibitory activity of ethanol extracts and different solvents fractions of Zataria multiflora, *Salvia mirzayanii* and *Otostegia persica* (Lamiaceae) was evaluated against α- glucosidase. Among the fractions, the ethyl acetate fractions of Z. *multiflora* ($IC_{50} = 0.35 \pm 0.01$ mg/mL), the petroleum ether fraction of S. *mirzayanii* ($IC_{50} = 0.4 \pm 0.11$ mg/mL) and the ethyl acetate fractions of O. persica ($IC_{50} = 0.5 \pm 0.16$ mg/mL) showed the highest inhibitory activity in comparison with acarbose ($IC_{50} = 7 \pm 0.19$ mg/mL) (25). In another study, an aqueous extract of Z. multiflora exhibited a percentage of inhibitory more than 80% against α- glucosidase in vitro model (26). This extract ameliorated insulin-resistance in insulin-resistant rats through a different mechanism including insulin-like effect, an increase in the expression of PPARγ protein and adiponectin (27). The hydroalcoholic extract of this plant increased the insulin levels, modified the liver enzymes, damage caused by oxidative stress, inflammation and hyperglycemia (28). The flavonoids, apigenin, luteolin and 6-hydroxyluteolin (29), luteolin-7-O-glucopyranoside, apigenin-7-O-rutinoside and luteolin-7-O-rutinoside were determined as the active constituents of Z. multiflora (30). Some of these flavonoids showed potent free radical scavenging activity (31) and strong α-glucosidase inhibitory effects (7, 32). In the present study, the petroleum ether fraction of M. aucheri exhibited the most active enzyme inhibition ($IC_{50} = 217.0 \pm 5.5$ µg/mL or 0.21 mg/mL) compared to that of acarbose ($IC_{50} = 173.5 \pm 1.3$ or 0.17 mg/mL). It indicates that the petroleum ether fraction can be considered as a potential source of natural α-glucosidase and it could be a suitable candidate for isolation of natural α-glucosidase inhibitors in the future.

There was a positive correlation between TPC and α-glucosidase inhibitory and antioxidant activity especially in the FRAP assay (Table 2). The Pearson correlation coefficient value of + 0.962 confirms that there was a very strong positive-correlation between the two FRAP and TPC variables. In addition, the samples with higher total phenol contents showed higher free radical scavenging power. The results of the present study are supported by previous studies (33, 34). Also, a positive and significant correlation was observed between TPC and α-glucosidase (0.649) and DPPH (0.591) values while there was no

Table 1. Antioxidant activity and total phenol contents and inhibition activity of solvent extracts and fractions of M. aucheri against α-glucosidase enzyme

Extract/ LLE fractions	α- glucosidase IC_{50} (µg/mL)	DPPH IC_{50} (µg/mL)	FRAP	TPC
MeOH	1263.2 ± 2.5*	158.9 ± 2.5*	118.5 ± 2.1*	82.8 ± 3.7
EtOH	1141.6 ± 10.0*	190.7 ± 6.6*	148.9 ± 4.6*	76.6 ± 0.6
EtOH/ EtOAc	599.9 ± 2.2*	66.6 ± 1.5*	367.4 ± 2.3*	155.1 ± 11.2*
EtOH/ DCM	977.4 ± 12.9*	-	57.0 ± 0.8*	45.4 ± 1.3**
EtOH/ petroleum ether	217.0 ± 5.5**	-	-	-
Acarbose	173.5 ± 1.3*	-	-	-
Quercetin	-	3.1 ± 0.1*	-	-

a) DPPH IC50 (µg samples /1 mL 10-4 M DPPH, b) FRAP: mg FeSO4/ g extract, c) TPC: Total phenol contents (mg Gallic acid /1g of extract), Mean ± SE, **P <0.05, *P < 0.0001

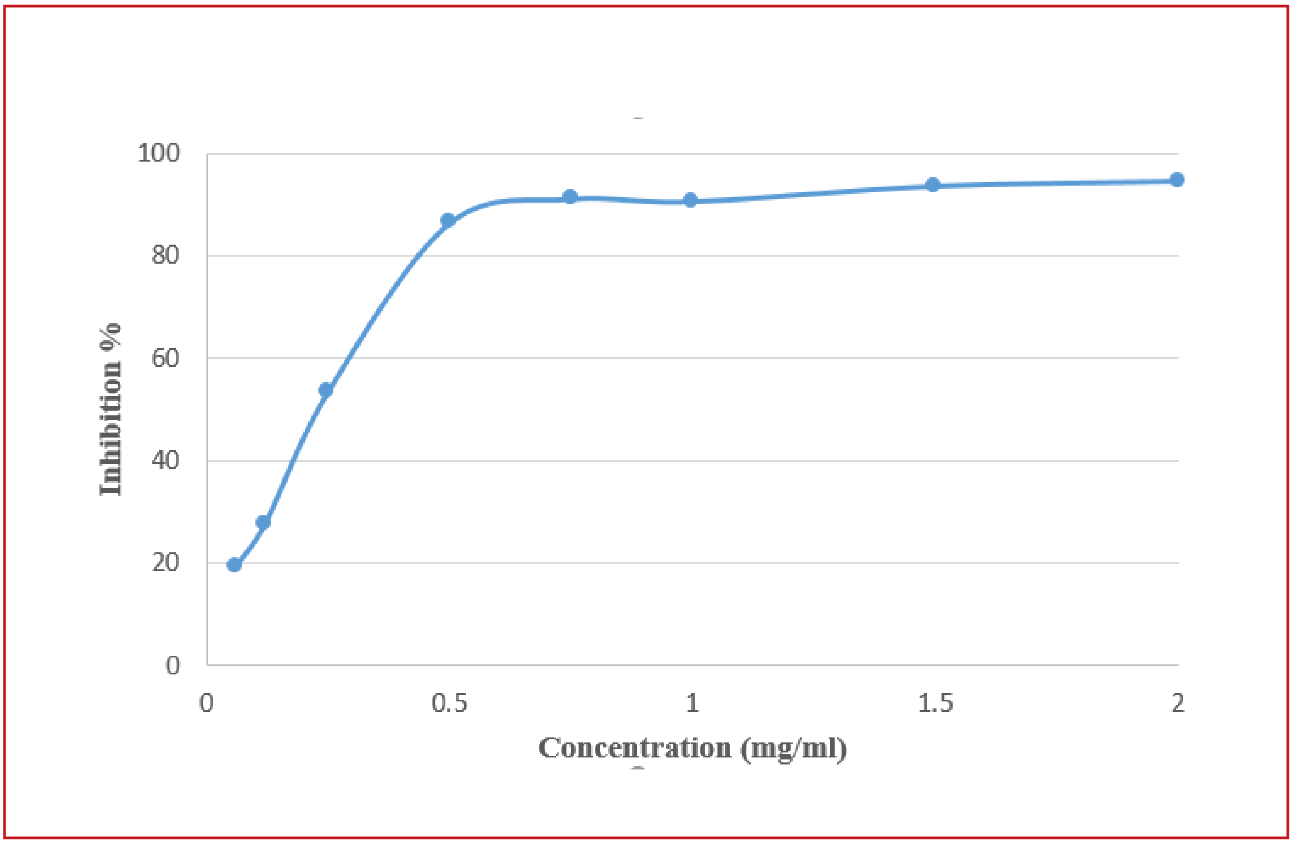

Chart 1. Inhibition percentage of petroleum ether fraction at different concentrations (0.06- 2.00 mg/ml)

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Table 2. Pearson's correlation coefficients between total phenolics and biological activities

	TPC	DPPH	FRAP	Enz. Inh.
DPPH	0.591	1		-
P value	0.043	-		-
FRAP	0.962	0.728	1	-
P value	<0.001	0.007	-	-
Enz. Inh.	0.649	-0.076	0.527	1
P value	0.023	0.815	0.078	-

TPC: Total phenol content, DPPH and FRAP: antioxidant assay, Enz. Inh.: enzyme inhibition

Table 3. Inhibition activity of isolated compounds from DCM extract of M. aucheri against α-glucosidase enzyme

Compounds	IC50 (µM)
genkwanin (1)	110 ± 0.4
5-hydroxyl-7, 4′-dimethoxy flavone (2)	320 ± 2.1
stigmasterol and β-sitosterol (3, 4)	NA
Acarbose	217 ± 0.5

NA: Not active, Final concentrations (15.6-250 µg/mL), Mean ± SE, n=3

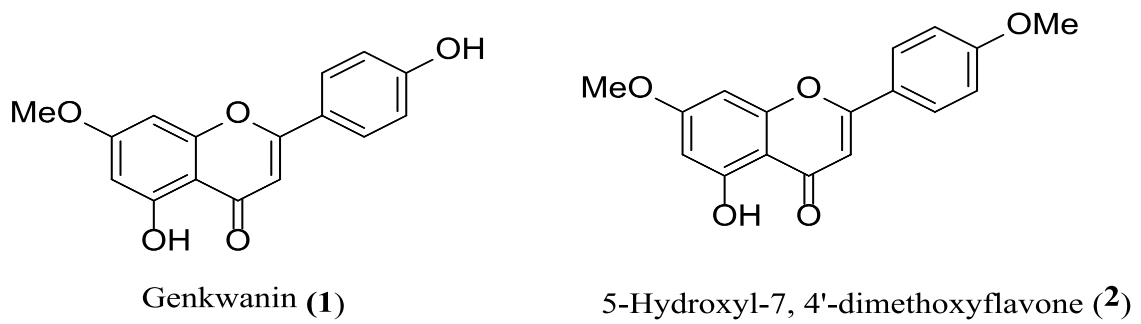

Figure 1. The chemical structures of the flavonoids with α- glucosidase inhibitory activity

correlation between α-glucosidase inhibition assays with DPPH assay (-0.076, P > 0.05).

Genkwanin (1) showed higher while 5-hydroxyl-7, 4′-dimethoxy flavone (2) exhibited lower α-glucosidase inhibitory activity compared to that measured for acarbose while phytosterols of stigmasterol and β-sitosterol mixture (3, 4) were inactive in the above enzyme inhibition test (Table 3). So far there was no phytochemical report on M. aucheri in the literature except that we have reported recently (21).

The chemical structure of apigenin is very similar to 1 (7-methyl apigenin) which was introduced as an antidiabetic agent. Apigenin protects pancreatic β-cells from oxidative cell damage caused by 2-deoxy-D-ribose (dRib) (35) and inhibited α-glucosidase enzyme (36). In addition to apigenin with pronounced α-glucosidase inhibition activity (IC_{50}= 82 ± 6 μM); the related flavonoids, luteolin (IC50= 46 ± 6 μM), chrysoeriol (IC_{50}= 156 ± 5 μM), morin (IC_{50}= 32 ± 2 μM) and quercetin (IC_{50}= 15 ± 3 μM) exhibited high activities compared to that reported for acarbose (IC_{50}= 607 ± 56 μM) (7). Luteolin (3′-hydroxy apigenin) displayed higher activity than apigenin which seems to be due to the presence of an extra hydroxyl group. On the other hand, chrysoeriol (3′-methoxy apigenin) showed lower activity than that measured for apigenin. In addition to the above flavones, quercetin, a flavonol, was introduced as one of the most active α-glucosidase inhibitors (7). The above-mentioned structure activity relationship (SAR) studies of flavonoids proved the positive effect of hydroxylation at C3, C5, C7, C8, C3 and C4′ on their enzyme inhibition. Therefore, the presence of a free hydroxyl group at C4’ in 1 is consistent with its higher enzyme inhibitory activity compared to that of compound 2 (Table 3, Figure. 1).

In addition to α-glucosidase inhibition potential of compounds 1 and 2, they showed radical scavenging and pro-oxidant activity determined by ABTS) 2,2’-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and FRAP assays, respectively (37). Compound 1 has shown scavenging activity against peroxynitrite (ONOO−) (38) and exhibited potent antiviral activity against African Swine Fever Virus (ASFV) which suggested it as a candidate for antiviral drugs (39). Finally, this constituent showed antitumor and immunomodulatory activity on colorectal cancer (40).

Therefore, M. aucheri plant with high levels of phenolic compounds such as bioactive flavonoids and good antioxidant and inhibition of α-glucosidase enzyme can be considered a candidate for further studies to isolate bioactive compounds.

Conclusion

We found a positive correlation between the amounts of TPC, antioxidant potential and α-glucosidase enzyme inhibitory activity of the plant's extracts and their resulting fractions. However, no correlation was measured between the antioxidant and α-glucosidase enzyme inhibitory activity. Since both antioxidant and α-glucosidase enzyme inhibitory activity are beneficial for the treatment of diabetic disease or its complications, we suggest here the ethyl acetate fraction as a potential natural drug candidate for further investigation. On the other hand, since at least two of the methoxylated flavonoids; 1 and 2 are isolated from a non-polar fraction of the plant, they might be the reason for the high activity of the petroleum ether fraction of the plant ethanol extract.

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Conflict of interest

The authors declare that they have no conflict of interest.

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