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STUDY ON THE ANTI-HYPERGLYCEMIC EFFECT OF *HIBISCUS TILIACEUS* L. LEAVES IN OBESE/DIABETIC (+DB/+DB) MICE

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Summary

Study on the Anti-Hyperglycemic Effect of *Hibiscus tiliaceus* L. Leaves in Obese/Diabetic (+DB/+DB) Mice

Diabetes mellitus is considered as the main cause of burning the global health system every year. This study aims to contribute to this growing area of research by investigating the anti-hyperglycemic effect of 70% ethanol extract from *Hibiscus tiliaceus* L. leaves at the oral doses of 0.52 g/kg and 1.04 g/kg (equivalent to 2.5 g and 5 g absolute dry weights of raw materials per kilogram mouse body weight) in the high fatty diet-induced obesity mice with diabetes mellitus by streptozotocin agent (HFD+STZ). After 14 days of oral treatment, the results showed that the 70% ethanol *H. tiliaceus* L. leaves extract at the oral doses of 0.52 g/kg and 1.04 g/kg decreased the blood glucose level significantly in comparison with pathological control (HFD+STZ). This effect of the 70% ethanol *H. tiliaceus* L. leaf extract at the oral dose of 1.04 g/kg was better than the reference drug metformin (500 mg/kg). Moreover, it reduced triglyceride in the plasma significantly in comparison with pathological control (HFD+STZ). According to this study, there was only one 70% ethanol *H. tiliaceus* L. leaves extract at the oral dose of 1.04 g/kg lowed total cholesterol significantly as compared with pathological control (HFD+STZ). All these that *Hibiscus tiliaceus* L. leaves extract might be potentially in the supportive treatment for the non-insulin-dependent diabetes mellitus in obesity patients in the future.

Keywords: *Hibiscus tiliaceus* L. *Leaves, Anti-hyperglycemic, Diabetes mellitus, High-fat diet, Streptozotocin.*

1. Introduction

The rapid development of modernization, urbanization, and accelerated socio-economic growth favored an improved living standard but a more stressful and sedentary lifestyle and unhealthy dieting habits in most parts of the world. Especially in the last two decades, obesity has become a global pandemic threatening people's lives by affecting almost every organ system and is now a severe public health problem as one of the most common non-communicable diseases (NCDs). With no exception, all countries are now being affected by obesity, and this impact is predicted to be even more prominent during the current decade, resulting in more lost years of a healthy life, disability, and death. Hence, it is urgent to make effective and decisive actions to hinder the rise in the prevalence of obesity to prevent and treat obesity and other obesity-related comorbidities, among which type 2 diabetes mellitus (T2DM) is a health issue growing at an alarming speed in all regions as another global health emergency of the 21st-century. The booming increase in the prevalence of obesity in all age groups is one of the main culprits of the exponential growth of the population of T2DM [1].

Hibiscus tiliaceus L. is a scientific name of the plant that depends on the *Hibiscus* genus, Malvaceae family. It is also called by the

Vietnamese name, "Tra làm chiếu". It is determined a bunch kinds of secondary metabolites in this plant such as alkaloids, flavonoids, tannins, terpenoids, steroids, and so on from many different parts of the plant. Le et al (2021) demonstrated that the presense of flavonoid glycoside (astragalin or kaempferol-3-O-glucoside, isoquercitrin, rutin, and trans-tiliroside) in this medicinal plant source [2]. Many studies have investigated the hypoglycemic effect on mice based on the pharmacological activity of the *Hibiscus* genus in many decades. Ragnunathan and Sulochana (1994) demonstrated a significant hypoglycemic effect from the *Hibiscus vitifolius* flowers. In 2001, Sachdewa et al. showed that a dose of 250 mg/kg body weight of rats for the *Hibiscus rosa-sinensis* leaves extracts with the dramatically reduced blood glucose levels in the streptozotocin-induced rats. Furthermore, Kumar et al (2010) demonstrated the effectiveness of antidiabetic and hypolipidemic activities of the methanol *Hibiscus tiliaceus* flower extract in STZ-treated rats leading to the reduction of total cholesterol, triglycerides, and weight loss in rats [3],[4]. Therefore, the aim of this study is to investigate the hypoglycemic effect of the 70% ethanol from *H. tiliaceus* L. leaves extract in the HFD/STZ mice model.

2. Materials and methods

2.1. Plant materials

Hibiscus tiliaceus L. leaves were collected from Thanh An, Can Gio Town, Ho Chi Minh City, Vietnam in December 2022. It was conserved in the Research Center of Ginseng and Medicinal Materials in Ho Chi Minh City (botanical sample and gene conservation, code: TB-3182). The material was extracted with 70% ethanol at room temperature for 72 hours in percolator apparatus (ratio 1:20, w:v). The ethanol extracts were collected at a rate of 2 ml/min and concentrated using a rotary evaporator at 60°C under reduced pressure to obtain crude ethanol extracts (HTL). The yield of HTL viscous extracts was 20.82%. The humidity of HTL semi-solid extracts was 15.51%.

The pharmacology experimental doses of HTL are 0.52 g/kg and 1.04 g/kg body weight mice (equivalent to 2.5 g and 5.0 g materials per kilogram body weight mice) (based on the folk experience and guidelines for Preclinical and Clinical Study of Traditional and Natural Medicines" approved by Ministry of Healthy-Vietnam (attached with Decision No.141/QĐ-K2ĐT dated on 27/10/2015)), which referred to the experimental dose using for human based on the extraction efficiency (20.82%, except for humidity).

2.2. Animals

Swiss albino male mice (18 ± 2 g, aged 5-6 weeks) were used for the experiments. Mice was purchased from the Institute of Vaccines and Medical Biological Products in Nha Trang City (IVAC). The manipulation of animals were conducted according to "Guidelines for Preclinical and Clinical Study of Traditional and Natural Medicines" approved by the Ministry of Health in Vietnam (attached with Decision No.141/QĐ-K2ĐT dated 27/10/2015). Tap water and standard laboratory food were made available ad libitum. The mice were allowed to acclimatize for a week prior to the experiment. The oral or injection volume was 10 mL/kg b.w.

2.3. Chemicals- Reagents

Streptozotocin (Sigma, USA); Glucophage 500 mg (Metformin hydrochloride 500 mg, France); 96% ethanol (OPC-Vietnam); Kit-test for the quantitative of Glucose, Cholesterol, and Triglycerides (HUMAN, Germany). All other chemicals were of analytical grade.

2.4. Experimental design

Performing the evaluation on the diabetic-induced mice with the high-fat diet (HFD) (the components of high-calories diet consists of standard food (IVAC), egg yolk, pork-derived lipid) and the diabetogenic agent (streptozotocin - STZ) facilitates to produce the obesity animal model and insulin resistance that mimic to non-insulin dependent diabetes mellitus. The standard food was provided by the Institute of Vaccines and Medical Biological Products in Nha Trang City. It contains a total protein of 21%, lipid of 7 %, fibrous of 6 %, total minerals of 8%, several acid amines, and some essential vitamins. Whilst that, the composition of a high-fat diet contains 40.1% carbohydrate, 19.9% protein, 30.7% lipid, and other substances. The experiment is divided into three stages in below:

First stage: mice are randomly separated into two experimental groups (n = 8). The control group (HFD (-)) is fed standard food (IVAC) while that of the treatment group is induced by a high-fat diet (HFD (+)). Furthermore, the plasma total cholesterol and

triglycerid are measured after 2, 4, 6, and 8 weeks. These parameters include body weight of mice (%), plasma total cholesterol (mg/dL), and plasma triglyceride (mg/dL) which were an evidence-based criteria for the selection of time causing obesity.

Second stage: carrying out the investigation of diabetic-induced mice on the obesity model (HFD)

Investigation of experimental doses of STZ causing hyperglycemia in obese mice in the following treatment groups (n=8):

HFD (-): Biological control group, normal healthy mice.

HFD (+): The obesity mice were not injected with streptozotocin.

HFD (+) vs STZ (50 mg/kg, 1st): The obese mice were injected once with 50 mg/kg STZ administration, i.p.

HFD (+) vs STZ (50 mg/kg, 2nd): The obese mice were injected twice with 50 mg/kg STZ administration, i.p (The interval between the two occasions was seven days).

HFD (+) vs STZ (100 mg/kg, 1st): The obese mice were injected once with 100 mg/kg STZ administration, i.p.

After 7 days STZ- induced, evaluation of the plasma glucose of the groups to select the dosage used for inducing a diabetic rodent model. The plasma glucose level is > 126 mg/dL (in fasting) or > 200 mg/dL (non-fasting) is accepted for research animal model, which is followed by Guidelines for Preclinical and Clinical Study of Traditional and Natural Medicines (Ministry of Health 2015, Vietnam).

Investigation of the hypoglycemic activity of metformin dosages in the following treatment groups (n=8):

HFD (-): Biological control group, normal healthy mice

HFD (+): The obesity mice were not injected with streptozotocin

HFD (+) vs STZ (100 mg/kg): The obese mice were injected once with 100 mg/kg STZ administration, i.p.

HFD+STZ (100 mg/kg, i.p)+ Metformin (250 mg/kg, p.o): The obesity mice were injected 100 mg/kg STZ additional to 250 mg/kg metformin p.o administration

HFD+STZ (100 mg/kg, i.p)+ Metformin (500 mg/kg, p.o): The obese mice were injected 100 mg/kg STZ additional to 500 mg/kg metformin p.o administration.

After 14 days of metformin- oralled, an evaluation of the plasma glucose of the groups to select the dosage used metformin which showed the hyperglycemia effected comparison with HFD (+) vs STZ (100 mg/kg) group.

Third stage: *Evaluation of the hypoglycemia effect of HTL in the following treatment groups (n=8):*

Table 1. Experimental design for evaluation of the hypoglycemia effect of HTL

Groups (n=8)	The samples
HFD (-)	Distilled water
HFD (+)	Distilled water
HFD (+) vs STZ (100 mg/kg)	Distilled water
	HTL (0.52 g/kg)
	HTL (1.04 g/kg)
	Metformin (500 mg/kg)

One hour after oral the samples on the 14th day (on the 7th week of the experiment, after mice were caused HFD with 4 weeks and - induced STZ (100 mg/kg), i.p.) with a week and oralled the samples with 2 weeks), tail vein blood of groups was taken to determine glucose, triglyceride, cholesterol contents in plasma (according to kit Human Co. Ltd., Germany) [4],[5].

2.5. Data analysis

The results were expressed in terms of mean ± SEM (Standard error of the mean) using MS Excel 2016 software. Data were analyzed by Systat statistical software (version 3.5, Inc. SigmaStat) using a t-test and One-way ANOVA followed by the Student-Newman-Keuls test.

3. Results

3.1. Results of surveying obesity model

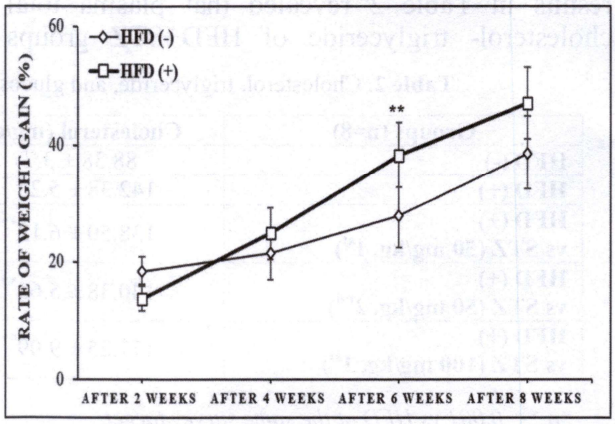

Fig. 1. Rate of weight gain of the groups after 2, 4, 6, and 8 weeks. ** *p<0,01 vs HFD (-) at the same time*

Fig1 illustrates the effect of a high-fat diet (HFD) on the body weight of mice. The rate of

weight gain of HFD (+) showed no significant differences as a comparison with HFD (-) at the two weeks. It is obvious that the increased the rate of weight gain in the HFD (+) group was higher than the rate of weight gain in the HFD (-) group from the 4 weeks to the 8 weeks; however, this was only the statistically significant at the 6 weeks ($p=0.007$).

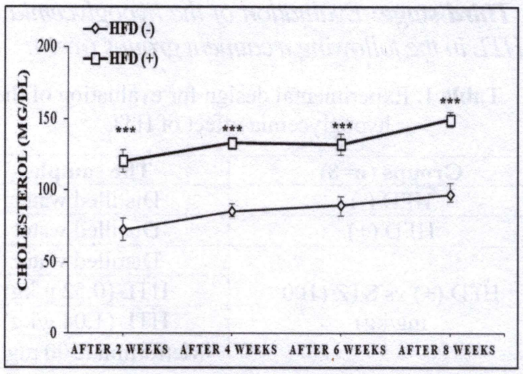

Fig. 2. Cholesterol of the groups after 2, 4, 6, and 8 weeks. $^{***}p<0.001$ *vs HFD (-) at the same time*

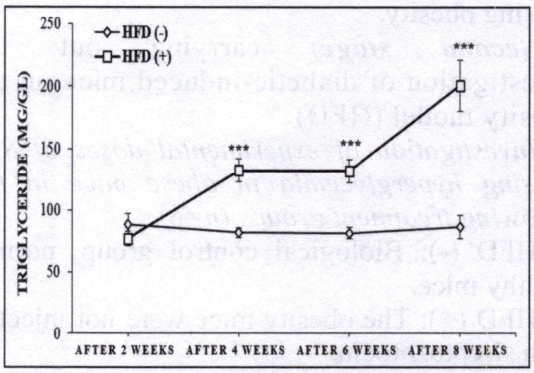

Fig. 3. Triglyceride of the groups after 2, 4, 6, and 8 weeks. $^{***}p<0.001$ *vs HFD (-) at the same time*

In parallel to the evaluation of the rate of weight gain, this study also investigated some biochemical parameters including the plasma total cholesterol level and the plasma triglycerid level. The findings of Fig. 2 and Fig. 3 showed that the plasma total cholesterol and triglyceride of the HFD (+) group raised (47.77% - 56.98%; 61.83% - 131.60%; respectively) significantly as compared with the HFD (-) group after 4, 6, and 8 weeks ($p<0.001$). So, this study was timed after 4 weeks of high-fat feeding to induce hyperglycemia by STZ.

3.2. Results of surveying diabetes in mice with obesity model

Investigation of experimental doses of STZ causing hyperglycemia in obese mice: The results in Table 2 revealed that plasma total cholesterol- triglyceride of HFD+STZ groups (STZ induced) increased significantly as compared to HFD (-) and HFD (+) ($p<0.001$). In parallel to plasma glucose of HFD (+) vs STZ (50 mg/kg, 2nd) and HFD (+) vs STZ (100 mg/kg, 1st) rised (54.16%-93.33%) significantly as compared to HFD (-) and HFD (+) ($p<0.001$) while plasma glucose of HFD (+) vs STZ (50 mg/kg, 1st) group was no significantly as compared to HFD (-) and HFD (+). Besides, the plasma glucose of HFD (+) vs STZ (100 mg/kg, 1st) was higher than HFD (+) vs STZ (50 mg/kg, 2nd) group. Moreover, the rate of mice-induced STZ number with plasma glucose over 126 mg/dL HFD (+) vs STZ (100 mg/kg, 1st) group was higher than HFD (+) vs STZ (50 mg/kg, 2nd) group. In summary, this study used STZ (100 mg/kg, 1st, i.p.) to induce hyperglycemia in obese mice.

Table 2. Cholesterol, triglyceride, and glucose of the groups in obese/diabetic (+db/+db) mice

Groups (n=8)	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)
HFD (-)	88.38 ± 3.77	94.25 ± 7.15	110.13 ± 3.43
HFD (+)	142.38 ± 5.23***	145.25 ± 7.20***	104.13 ± 7.79
HFD (+) vs STZ (50 mg/kg, 1st)	138.50 ± 6.12***	125.38 ± 3.78**	115.38 ± 5.81
HFD (+) vs STZ (50 mg/kg, 2nd)	140.38 ± 5.65***	139.63 ± 11.13***	134.63 ± 2.89*** **(54.16%)**
HFD (+) vs STZ (100 mg/kg, 1st)	177.25 ± 9.09***	149.88 ± 11.47***	175.88 ± 15.88*** **(93.33%)**

*$p < 0.001$ *vs HFD at the same survey target.*

Investigation on the hypoglycemic activity of metformin dosages: The results Fig4. showed that the groups- oralled metformin wasn't hypoglycemia effect after 7 days of treatment. After 14 days of treatment, the oralled metformin (500 mg/kg) group was significantly hypoglycemia effect in comparison with HFD (+) + STZ (100 mg/kg) ($p=0.003$). It decreased the

plasma glucose which was better than metformin (250 mg/kg) ($p<0.001$). Moreover, metformin (500 mg/kg) was used to give the plasma glucose levels back to normal value as comparison with HFD (-) ($p=0,356$) in obese/diabetic (+db/+db) mice (HFD (+) vs STZ (100 mg/kg)).

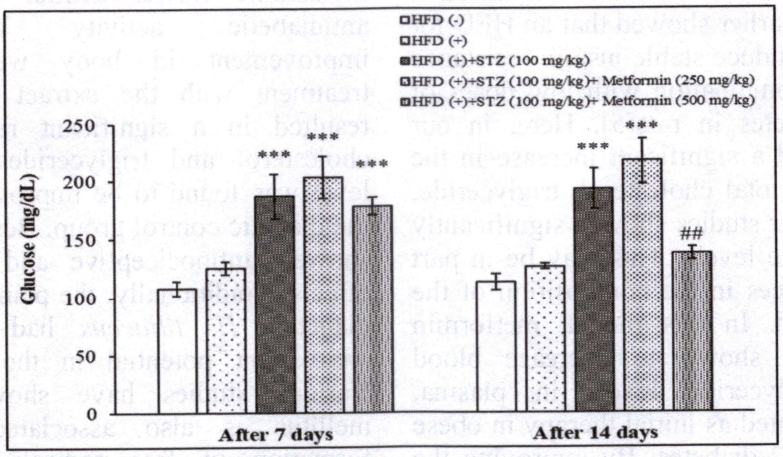

Fig. 4. Glucose of the groups. $^{***}p<0.001$ *vs HFD (-),* $^{\#\#}p<0.01$ *vs HFD (+) + STZ (100 mg/kg)*

3.3. Effect of HTL on plasma cholesterol-triglyceride and glucose level in obese/diabetic (+db/+db) mice

According to Table 3 and Fig 4, pathological control (HFD (+) vs STZ (100 mg/kg)) showed a significant increase of 56.89%, 43.26% in plasma glucose as compared to HFD (-) and HFD (+), respectively ($p<0.001$). Metformin administered in obese/diabetic (+db/+db) mice at the dose of 500 mg/kg exerted a significant reducing effect (18.97%) on plasma glucose as compared to pathological control ($p=0.006$). Moreover, metformin (500 mg/kg) also significantly decreased by 19.05% in plasma triglyceride as compared to HFD (+) ($p=0.026$).

Table 3. Cholesterol, triglyceride, and glucose of the groups

Groups (n=8)		Cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)
HFD (+) vs STZ (100 mg/kg)	**HFD (+)**	148.00 ± 4.97	133.50 ± 5.43	125.13 ± 1.56$^{\#\#\#}$
	HTL (0.52 g/kg)	131.75 ± 6.41	113.88 ± 9.64$^{\#\#}$	139.88 ± 8.26$^{\#\#}$
	HTL (1.04 g/kg)	111.38 ± 6.06$^{\#\#\#}$	113.38 ± 8.47$^{\#\#}$	118.75 ± 7.44$^{\#\#\#}$

$^{\#}p<0.05$ *vs HFD (+) + STZ (100 mg/kg);* $^{\#\#}p<0.01$ *vs HFD (+) + STZ (100 mg/kg);* $^{\#\#\#}p<0.001$ *vs HFD (+) + STZ (100 mg/kg).*

HTL (0.52 g/kg- 1.04 g/kg) exerted a significant decrease (21.97%- 33.75%) in plasma glucose as compared to pathological control ($p= 0.004; p<0.001;$ respectively). The effect of HTL on plasma glucose was similar to that of metformin (500 mg/kg) ($p=0.646; p=0.119;$ respectively). Besides, HTL also showed triglyceride-lowering effect (24.21%- 24.54%) as compared to pathological control ($p=0.026; p=0.007;$ respectively) which was similar to that of metformin at the oral dose of 500 mg/kg ($p=0.467; p=0.963;$ respectively). Meanwhile, the cholesterol-reducing effect was only showed by HTL (1.04 g/kg) in obese/diabetic (+db/+db) mice.

4. Discussion

The present study shows the induction of diabetes in a rat model using STZ alone (T1DM) and in combination with high fat diet (T2DM). High-fat feeding will lead to obesity, hyperinsulinemia, and altered glucose homeostasis due to insufficient compensation by the beta cells of the pancreatic islets. The T2DM model in our study typifies human T2D progression with associated dyslipidemia, insulin resistance, prediabetes, and diabetes. Diabetes is a complicated, heterogenic, and polygenic disease, while T1DM is associated with destruction in the pancreatic acini cells commonly *via* auto-immune mechanism. T2DM is associated with the development of insulin resistance by target organs and decreased insulin production from pancreatic beta cells11. A single dose of 100 mg/kg of STZ as seen in this study was associated with the pancreatic cells.

Accordingly, STZ induced after HFD causes less destruction of the pancreas, this animal model portraits the same characteristics and mimics the pathogenesis and clinical features of human T2D. Some researchers earlier showed that an HFD for 2–7 weeks would induce stable insulin resistance and the HFD in combination with low doses of STZ induces diabetes in rats[5]. Here, in our study, we observed a significant increase in the blood glucose and total cholesterol, triglyceride, although some other studies showed significantly elevated triglyceride levels. This may be in part due to the differences in the composition of the high-fat diets used. In this model, metformin (500 mg/kg) was shown to decrease blood glucose and triglyceride levels in plasma. Metformin is indicated as initial therapy in obese patients with type 2 diabetes. By improving the sensitivity of cells to insulin, metformin reduces hepatic glucose production and increases glucose uptake in muscle and other peripheral tissues [6].

This study demonstrated that treatment of HTL, as well as metformin reduced plasma glucose, cholesterol, and triglyceride in obese/diabetic (+db/+db) mice. *H. tiliaceus* L. (Malvaceae), commonly known as "bola" is a mangrove plant growing in tropical Asia and abundant in forests. In folk medicine, the leaves of this plant are used to treat fevers and soothe coughs, ulcers, wounds, and various skin diseases. In the Indian system of medicine, *H. tiliaceus* had been used as febrifuge, laxative, resolvent, and emollient. An infusion of leaves is employed to wash ulcers and wounds. The fruit juice is rubbed on the skin to cure weakness. The flower extract also exhibited significant reducing power and free radical scavenging effect on hydroxyl, superoxide, and hydrogen peroxide radicals. The present study was carried out to evaluate antidiabetic and hypolipidemic activities of *H. tiliaceus* methanolic flowers extract in streptozotocin-induced diabetic Wistar rat by administering graded oral doses (250 and 500 mg/kg body weight) for 21 days [4]. Kumar S. et al. (2010) demonstrated that *H. tiliaceus* methanolic flower extract showed significant antidiabetic activity with significant improvement in body weight. Daily oral treatment with the extract for 21 days also resulted in a significant reduction in serum cholesterol and triglycerides. HDL-cholesterol level was found to be improved as compared to the diabetic control group. Besides, the plant also showed antinociceptive and anti-inflammatory effects. Traditionally, the plant has been used for diabetes. *H. tiliaceus* had showed *in vitro* antioxidant potential in the previous reports. Various studies have shown that Diabetes mellitus is also associated with increased formation of free radicals and decrease in antioxidant potential. It is accepted that oxidative stress results from an imbalance between the generations of oxygen-derived radicals and the organism's antioxidant potential [4]. So, the antidiabetic properties of *H. tiliaceus* might be due to the antioxidant effect of the plant. Until now, the exact mechanism of action of reduction of blood glucose levels after administration (p.o.) of the extracts is not clear. The extracts should further be subjected to bioactivity-guided drug discovery to isolate a lead compound responsible for this activity.

5. Conclusion

The extracts from *H. tiliaceus* leaves at the oral doses 0.52 g/kg- 1.04 g/kg (equivalent to 2.5 g and 5 g of raw materials/kg BW) were effective in reducing plasma glucose and triglycerides, total cholesterol levels in obese/diabetic (+db/+db) mice.

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