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Morinda citrifolia (Noni) changes the expression of AdipoR2 mRNA in hepatic tissue of rats with nonalcoholic fatty liver disease (NAFLD)

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Abstract

Morinda citrifolia (Noni) have different therapeutics and hepatoprotective effects, the last effect is controversial; this study evaluated the effects of *Morinda citrifolia* leaf extract on expression of AdipoR2 mRNA in rats with nonalcoholic fatty liver disease (NAFLD). Four groups of wistar rats were utilized: C, NAFLD-Suc, NAFLD-Suc/Mc and NAFLD/Mc. The inducer of NAFLD was sucrose at 40% in water to drink. The Rats of the NAFLD-Suc/Mc and NAFLD/Mc groups received 200 mg of extract (2 weeks).

We observed difference in the body weight of rats intaking sucrose with respect to control group, during ten weeks of treatment. The NAFLD/Mc group showed decrease of liver weight, index liver and reversion of fatty liver with respect to NAFLD-Suc group. The same way, decrease expression of the gene AdipoR2 that codes for the receptor of adiponectine, which could be associated with the reduction of the content of triglycerides in the liver.

Keywords: *Morinda citrifolia*, non-alcoholic fatty liver disease, AdipoR2

1. Introduction

Morinda citrifolia Linn (family: Rubiaceae) is also known as “noni”, is distributed throughout the tropical and sub-tropical regions of the world such as French Polynesia and Hawaii. This plant have a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, antihelminths, analgesic, cardio-protective, hypotensive, immune enhancing, antiinflammatory, antioxidant, antiosteoporotic, insulinotropic, antidyslipidemic effects and hepatoprotective [1-9].

Morinda citrifolia prevents free-radical-induced oxidative-pathological events in liver by inhibiting inflammatory response and suppressing elevated liver enzyme activities; thus preventing consequent cell membrane damage [10], the Noni addition promotes hepatoprotection against alcohol-induced injury due to regulations of lipid homeostasis, antioxidant status and alcohol metabolism [11].

The claimed hepatoprotective properties on Noni still remain controversial, cases have been reported of *Morinda citrifolia*-associated liver injury, emphasizing its possible hepatotoxic effect [12, 13]. However, the association it is not clear, as no causal link could be established between the case of liver injury and ingestion of this plant [14].

No Alcoholic Fatty Liver Disease (NAFLD) is defined by the accumulation of fat in the liver or hepatic steatosis in the absence of excess alcohol consumption. The incidence of NAFLD worldwide has risen markedly in recent years in parallel with the increasing prevalence of global obesity and recent estimates reveal that there are approximately one billion cases worldwide [15].

Consumption of foods containing high fat and high sucrose, which are frequently associated with a “western diet,” account for the largest incidence of obesity and metabolic syndrome [16], which are strong risk factors for the development of fatty liver [17, 18].

Previous studies have investigated in human, animal and *in vitro* models the pathogenesis and molecular mechanisms through which some cytokines such as adiponectin influence on obesity, insulin resistance (IR), NAFLD and other components of metabolic syndrome (MS) [19, 20].

Adiponectin increases tissue fat oxidation, leading to reduced levels of fatty acids and tissue triglyceride content, thus enhancing insulin sensitivity in the liver and skeletal muscle, and plays a key role in slowing down the progression of NAFLD [21, 22].

Biological functions of adiponectin depend on not only the serum circulating concentration of hormone but also the expression level and function of its specific receptors including AdipoR1 and AdipoR2. AdipoR1 is most preferentially expressed in skeletal muscle and AdipoR2 is most preferentially expressed in liver [23].

AdipoR1 and AdipoR2 are ubiquitous receptors that modulate fatty acid metabolisms in the liver. This is demonstrated by the development of nonalcoholic steatohepatitis in rats fed a high-fat/high-cholesterol diet. Expression of AdipoR1/R2 is significantly reduced in NASH, which is associated with decreased AMPK α 1/ α 2 and PPAR α [24]. Consequently, the present study aimed to investigate the effects of *Morinda citrifolia* leaf extract on expression of AdipoR2 mRNA in rats with nonalcoholic fatty liver disease; as one of the possible targets of action of the extract on this pathology.

2. Materials and Methods

2.1 Collection and preparation of plant material

Dried *M. citrifolia* leaves were collected in San Bartolo Tuxtepec (18°05'35.69" N, 96°06'30.07" W) from the Papaloapan region of Oaxaca, Mexico. Taxonomic identification was performed by the curator of the Herbarium of CIIDIR-OAX, and a sample copy was deposited in their research laboratory for future reference. The plants were washed with tap water, dried on sheets of newspaper for 16 - 20 days, and then pulverized using a mechanical mill to obtain a powder that was subsequently hydrated [25].

2.2 Preparation of crude extract

To prepare the crude extracts, 50 g of dried and ground plants was added to 150 mL of distilled water in a flask and allowed to stand for 24 h. The solids were then separated from the liquid using filter paper and then discarded. The solvent was removed using reduced pressure on a rotary evaporator to obtain 15 g of crude extract. The crude extract was then stored in amber colored bottles at 8 °C until use.

2.3 Sucrose induced metabolic syndrome and fatty liver model

A total of 20 weaning male Wistar rats (Harlan Teklad), 21 days of age, were individually housed and maintained on a 12-h light/dark cycle at 25 °C. Animal maintenance and handling methods were in accordance with the 1985 National Institutes of Health Guide for the Care and Use of Laboratory Animals [26]. Animals were divided into two groups: the control group (C; n=5), which received a standard diet (Lab Diet CA.170481 Harlan Teklan Inc.), and the metabolic syndrome with NAFLD group (NAFLD-Suc; n = 15), which received the standard diet plus 40% sucrose, which was added to the drinking water; the animals were fed *ad libitum* for 10 weeks.

2.4 Experimental design

The animals with induced nonalcoholic fatty liver disease (NAFLD-Suc), described above, were divided into two groups. Both groups received a standard diet and *Morinda citrifolia* L extract (200 mg/kg) by oral cannula for 2 weeks; the first group (NAFLD-Suc/ Mc; n=5) received *M. citrifolia* extract and sucrose in the drinking water *ad libitum*, and the second group (NAFLD/Mc; n=5) received *M. citrifolia* extract without sucrose in the drinking water. Additionally, the fatty liver group received 40% sucrose in the drinking water (NAFLD-Suc; n=5) while the control group (C; n=5) received purified water without sucrose.

2.5 Blood samples

At the end of treatment period, blood samples from 18-hour-fasted animals were carefully collected to avoid hemolysis. The blood was centrifuged at 1086 \times g for 10 min, and serum samples were kept at -20 °C until analysis.

2.6 Analytical methods

Aspartate transaminase (AST) and alanine transaminase (ALT) were determined by colorimetric or enzymatic methods using an RA 10,000 Autoanalyzer (Bayer Diagnostics, Tarrytown, NY, USA), serum adiponectin levels were measured using an ELISA assay for rat adiponectin (Quantikine, R&D Systems).

2.7 Microscopic liver analysis

To assess liver damage, slices of organ were fixed for 24 h in 10% neutral buffered formalin. Sections (4–6 μ m) of the tissue was embedded in paraffin, and stained with hematoxylin and eosin (H–E) prior to microscopic examination [27]. Images were obtained using an Infinity Capture digital camera.

2.8 Real-time PCR Adipo R2 Gene

Total RNA Isolation

Total RNA was isolated from frozen rat liver. 150 mg of frozen-pulverized tissue was homogenized using a Tissumizer (Tekmar, Cincinnati, OH) in 1.5 mL of TRIzol™ Reagent (Invitrogen) and incubated at room temperature 5 minutes. Subsequently, 0.3 mL of chloroform was added and shaken by 15 seconds, after three minutes the samples were centrifuged at 12,000 \times g for 15 minutes at 4 °C and recovering the aqueous phase. The RNA was precipitated with 1 mL of isopropanol and incubated at room temperature for 10 minutes and recovered by centrifugation at 12,000 \times g for 20 minutes at 4 °C. The RNA was washed with ethanol at 75% and resuspended in RNase-free water and kept at -70 °C until use.

CDNA synthesis

The amount and quality of total RNA was determined spectrophotometry using NanoDrop 2000™ Spectrophotometer (Thermo Scientific) and 0.5 μ g for each sample of total RNA was normalized for cDNA synthesis. The reverse transcription synthesis reaction was performed using the iScript™ cDNA Synthesis kit (Bio-Rad). The cDNA was kept at -70 °C for later use.

Real-Time PCR

The relative expression of Adiponectine Receptor 2 (AdipoR2) was done in the StepOne™ Real Time PCR System (Life Technologies). The reaction was performed with IQ™ SYBR® Green Supermix (Bio-Rad), 1 μ L of cDNA and primers reported by Neumeier *et al.* 2006 [28] (AdipoR2uni 5'-GAAGGAGGGTCAACTCACCA-3', AdipoR2rev 5'-CATCAAGTTGGTGCCCTTTT-3'). Cycling conditions were as follows: three minutes at 95 °C for polymerase activation, 40 cycles of 15 seconds at 95 °C, 10 seconds at 60 °C and 10 seconds at 72 °C. β -actin gene expression was used as endogenous control, the primers sequence used for β -actin were: β -actin-uni 5'-TGGAATCCTGTGGCATCCATG-3 and β -actin-rev 5'-TAAAACGCGAGCTCAGTAACAG-3'. Melting curve analysis were performed after each RT-PCR in order to confirm that only one PCR products was detected. The relative expression of the target gene with respect to the internal control was calculated using relative quantification software provided by StepOne™ Real Time PCR System.

2.9 Data analysis

Data are presented as the mean \pm SD. Statistical significance was determined by analysis of variance, and Tukey's multiple range test was used for comparison of means ($P < 0.05$).

3. Results

3.1 Experimental model

The model with nonalcoholic fatty liver disease model, was generated by administration of drinking water containing 40% sucrose to male Wistar rats for 10 weeks. Figure 1 shows a significant difference in the body weight change for rats with (NAFLD-Suc group) and without (C group) sucrose ingestion. The body weight increase of NAFLD-Suc group was higher during all weeks with respect to control group ($p < 0.05$).

Table 1 shows liquid consumption, food and total calories intake for day in rats intaking sucrose in water drinking (NAFLD-Suc group) and rats of the control group. Differences were found in the total calories between both groups at 10 week compared with the first week of experimental treatment ($p < 0.05$).

3.2 Effects of *Morinda citrifolia* L extracts

After the rat sucrose-induced metabolic syndrome with nonalcoholic fatty liver disease model was established, the effects of dietary supplementation with *Morinda citrifolia* L leaf extracts were analyzed. Table 2 shown statistically significant differences between the liver weights of the C (6.60 ± 0.17 g) and NAFLD/Mc (5.53 ± 1.38 g) groups in comparison with NAFLD-Suc (8.16 ± 0.28 g) and NAFLD-Suc/Mc (8.36 ± 1.09 g) groups ($P < 0.05$). The C and NAFLD/Mc groups showed significantly lower index liver (2.60 ± 0.09 and 2.61 ± 0.10 respectively, than the NAFLD/Suc and NAFLD-Suc/Mc) groups (3.6 ± 0.10 and 4.0 ± 0.10 respectively). On the other hand, the C group showed levels significantly higher adiponectin/abdominal fat weight index (2.08 ± 0.10) than NAFLD-Suc (0.43 ± 0.01), NAFLD-Suc/Mc (0.45 ± 0.01) groups and to a lesser degree than the NAFLD/Mc (0.90 ± 0.02) group. No difference was observed in body weight between the four groups of the experimental design.

In relation with hepatic enzymes (AST and ALT) were found that AST/ALT index decrease in rats intaking sucrose without *M. Citrifolia* left extract (0.68 ± 0.01) which showed steatosis macrovesicular and microvesicular, hepatocellular injury and fibrosis; in the NAFLD-Suc/Mc and NFLD/Mc groups the AST/ALT index was > 1 .

As shown in figure 2, the liver lobular structure among rats in the control group did not reveal any noticeable histological changes (A), hepatic sections, from the NAFLD-Suc group showed a significant steatosis with hepatocellular injury (hepatocyte ballooning and apoptosis) and fibrosis. In the case of the NAFLD-Suc/Mc group the hepatocellular injury was lower than NAFLD-Suc group and without fibrosis (C) but NAFLD/Mc group presents an important reversion of the steatosis, hepatocellular injury and fibrosis compared with NAFLD-Suc and NAFLD-Suc/Mc groups (D).

Figure 3 shows the levels of gene expression AdipoR2 which are represented in the groups C (1.4 ± 0.07), NAFLD-Suc (4.68 ± 0.20), NAFLD-Suc/Mc (3.34 ± 0.10) and NAFLD/ Mc (3.02 ± 0.15), with significant difference between the four groups ($p < 0.05$).

4. Discussion

The objective of this study was to evaluate the effect of *Morinda citrifolia* leaf extract on expression of AdipoR2

mRNA in hepatic tissue an animal model of rats wistar with nonalcoholic fatty liver disease; this model was induced with high intakes of sucrose in water for drinking.

The consumption of sucrose in drinking water showed weight gain of NAFLD-Suc group as demonstrated by our results and those of others [29-31] (figure1). NAFLD-Suc group had reduced solid food consumption, but finally the total calories in the diet (solid diet plus sucrose in water) is higher with respect to control group at one and ten weeks of experimentation (Table 1). Is well known that changes in dietary macronutrient content are involved with the metabolism, insulin resistance and obesity development [32, 33] and many epidemiological studies have shown a positive relationship between higher intake of sweetened beverages and the risk of obesity [34].

In relation with intake of sucrose for the NAFLD-Suc group, this rats showed higher body weight; with regard to, Lomba *et al.*, 2009 suggest that high-sucrose diets appear to induce mitochondrial dysfunction in adipose tissue by down regulated of NDUFB6 gene, which may be related to greater weight gain and metabolic impairment by decreasing oxidative phosphorylation in the adipocyte [35].

In relation with body weights and ingestion of Noni the groups receiving the extract of *M. citrifolia* (Table 2) show no significant changes in body weight, which is consistent with reports in the literature that used another animal models e.g., hamsters, these studies has been reported that for a high cholesterol diet, there were no changes in body weight upon administration of noni juice [36, 37]; however, we us study shows a significant reduction in the liver weight and liver index in the NAFLD/Mc group with respect to NAFLD-Suc group ($p < 0.05$), these results are similar to those of other studies; Lin *et al.*, 2012 found that Noni juice supplementation decrements atherogenic index, and hepatic lipids in hamsters eating a diet high in fat; this events are associated with upregulated hepatic peroxisome proliferator-activated receptor-alpha and uncoupling protein 2 gene expressions, whit respect to levels of adiponectin and your relation to obesity with administration of Noni and accumulation of lipids. The results for the quotient; Adiponectin/abdominal fat weight, was increased in the NAFLD/Mc and NAFLD Suc/Mc groups in comparison with NAFLD-Suc group (Table 2); Kamada *et al.* 2007, 2008 in mice showed high levels of adiponectin may contribute to antagonize excess lipid storage in the liver and protects inflammation and fibrosis [38, 39].

In connection with the above is important to consider that polyphenols from the plant kingdom has been demonstrated hypolipidemic and anti-obesity effects through decreasing lipogenesis (up-regulations of SREBP-1c, ACC, FAS, etc.) or increasing energy expenditure (up-regulations of RXR- α , PPAR- α , UCP2, etc) [40, 41], previous studies also identified phenolic acids as the polyphenolic compounds, not flavonoids, in noni juice related with this effects [36, 37]. In addition, Chang *et al.* 2013 reported than consumption of this juice decreased liver lipid accumulation due to down-regulated de novo lipogenesis (SREBP-1c, ACC, and DAGT), up-regulated β -oxidation of fatty acids (PPAR- α and UCP2). Therefore the effect of *Morinda citrifolia* on the liver weight decrease, could be is related to high levels of polyphenolic compounds and increased adiponectin levels in the rodents.

With regard to the enzymatic liver profile high levels of enzymes responsible for the process of transamination (ALT and AST) are correlated with hepatocellular cytolysis, being appropriate indicators of liver damage; in this study a

significant decrease in the levels of AST and ALT under the effect of aqueous leaf extract *M. citrifolia* in NAFLD/Mc group compared to NAFLD-Suc group was obtained, these results support those reported by other research [10, 42]. Lin *et al.*, 2013 showed that Noni juice protects liver against a high-fat dietary habit via regulations of antioxidative and anti-inflammatory responses e.g. decrease of iNOS, COX-2, TNF- α and IL-1 β expressions in liver [11, 36].

Regarding the histopathological changes in the liver, the NAFLD group showed a significant liver injury (figure 2 B) in comparison with architecture normal the Control group, the result leads us to support the hepatoprotective effect of *Morinda citrifolia* to the development of the NAFLD.

Nayak *et al.*, 2011 studied the hypoglycemic and hepatoprotective activity of fermented fruit juice of *Morinda citrifolia* in diabetic rats, and founded that Noni juice potentiates the action of insulin directly or that it increase peripheral tissue sensitivity to storage hormone and to improve fat metabolism and as such reduce fatty accumulation in the liver [40].

In relation with AdipoR2 gene, which codes for the receptor of adiponectin, has connection with the pathology of fatty liver, since this hormone contribute to antagonize excess lipid

storage in the liver and protects inflammation and fibrosis.

We found that in the groups receiving the extract of *Morinda citrifolia* decreased AdipoR2 gene expression in the liver more than NAFLD-Suc group (Figure 2); De Oliveira *et al.*, 2011 demonstrated that the mRNA of AdipoR2 receptor increase in the liver tissue in rats with a High-fat diet and hyperglycemia; while adrenalectomy reduced AdipoR2 mRNA expression in liver [44]. Bullen *et al.*, 2007 found that High-fat feeding increase both expression of AdipoR1 and AdipoR2 in liver of rats, and this diet, is a factor that predispose humans to obesity and insulin resistance associated with decreasing adiponectin and increasing expression levels of the receptors mentioned [45].

In conclusion, these results showed a decrease in the expression of the gene AdipoR2 in liver under the effect of *Morinda citrifolia* leaf extract, and increase of the quotient: adiponectin/abdominal fat weight associated with reversion of hepatic steatosis in wistar rats.

5. Acknowledgements

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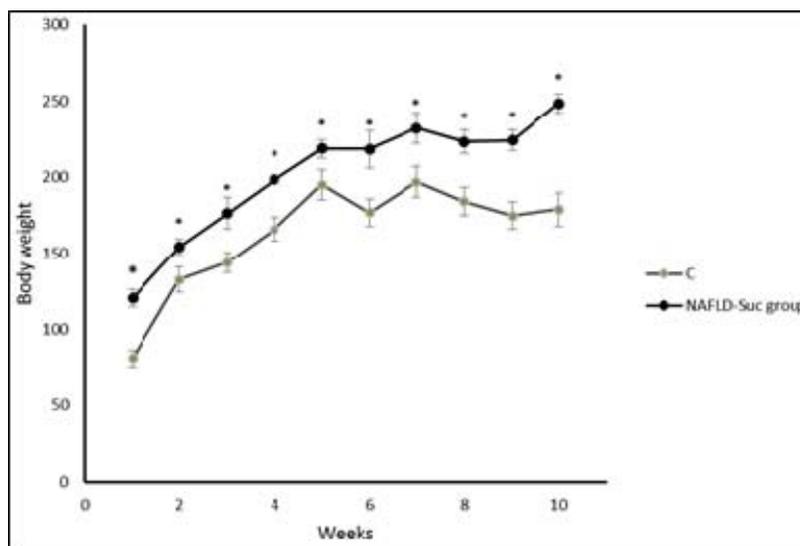


Fig 1: Body weights (g) of sucrose-fed and control rats. $P < 0.05$

Table 1. Liquid consumption, food and caloric intake for day in sucrose-fed (MS-FL group) and control (C groups) rats.

Parameters	C Group (n=5)		NAFLD-Suc Group (n=15)	
	1	10	1	10
Liquid consumption (ml/day)	31.25 \pm 5.05	51.20 \pm 1.09*	33.90 \pm 4.90	70.15 \pm 6.52*
Equivalent in Kcal in liquid consumption	----	----	54.24 \pm 7.84	112.27 \pm 10.44**
Food consumption (g/day)	25.00 \pm 2.24	33.33 \pm 5.65	14.75 \pm 1.20	15.40 \pm 5.78
Equivalent in Kcal in food consumption	95.00 \pm 8.50	126.65 \pm 21.47	56.05 \pm 4.56	58.52 \pm 21.96
Total calories (Kcal/day)	95.00 \pm 8.50	126.65 \pm 21.47*	110.29 \pm 12.40	170.79 \pm 32.40*

* $p < 0.05$ ** $p < 0.001$

Table 2. Body and liver weight and others parameters in rats fed experimental diets during 2 weeks.

Parameters	C Group (n=5)	NAFLD-Suc Group (n=5)	NAFLD-Suc/Mc Group (n=5)	NAFLD/Mc Group (n=5)
Body weight (g)	246 \pm 16 ^a	226 \pm 14 ^a	205 \pm 48 ^a	206 \pm 17 ^a
Liver weight (g)	6.60 \pm 0.17 ^a	8.16 \pm 0.28 ^b	8.36 \pm 1.09 ^b	5.53 \pm 1.38 ^c
Liver index	2.60 \pm 0.10 ^a	3.60 \pm 0.10 ^b	4.00 \pm 0.10 ^c	2.60 \pm 0.10 ^a
Adiponectin/abdominal fat weight	2.08 \pm 0.10 ^a	0.43 \pm 0.01 ^b	0.45 \pm 0.01 ^b	0.90 \pm 0.02 ^c
AST (UI/L)	79.50 \pm 2.20 ^a	57.76 \pm 4.74 ^b	96.67 \pm 6.70 ^c	74.33 \pm 4.30 ^a
ALT (UI/L)	44.00 \pm 5.00 ^a	85.00 \pm 6.90 ^b	66.70 \pm 5.20 ^c	33.00 \pm 4.10 ^d
AST /ALT	1.80 \pm 0.44 ^a	0.68 \pm 0.01 ^b	1.44 \pm 1.29 ^c	2.25 \pm 1.05 ^d

Liver index: (liver weight / body weight) (100) ^{a, b, c, d}, $P < 0.05$

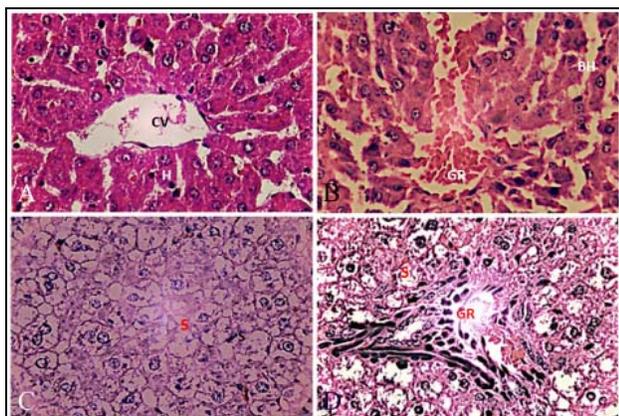


Fig 2: H & E 40 x. Liver sections from male rats. (A) C Group: Normal architecture of rat hepatic lobule. (B) NAFLD-Suc: Steatosis macrovesicular and microvesicular, hepatocellular injury and fibrosis. (C) NAFLD-Suc/Mc: Steatosis microvesicular. (D) NAFLD/Mc: Reversion of the steatosis, hepatocellular injury and fibrosis.

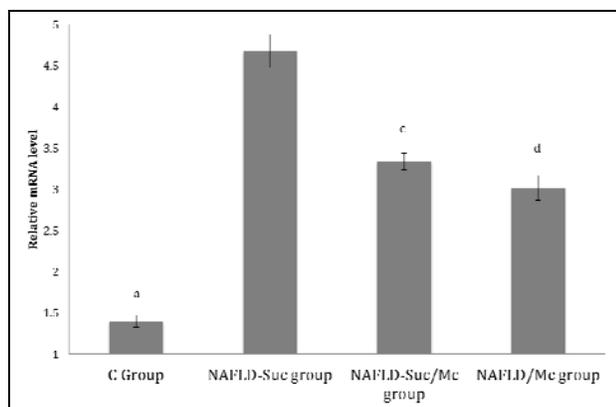


Fig 3: Relative expression of AdipoR2 gene in C group, NAFLD-Suc group, NAFLD-Suc/Mc group and NAFLD/Mc group. ^{a, b, c, d} $P < 0.05$

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