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Article

Cytotoxic and Mutagenic Effects and cell Viability of *Polybia sericea* Social Wasp Venom (Hymenoptera: Vespidae)

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ABSTRACT

The venom of social wasps can be a natural promising product for the development of drugs, since in recent decades studies have investigated its antimicrobial potential, anti-inflammatory, antioxidant, anticarcinogenic, among others for its future applicability in the pharmacological industry. Although some studies have already highlighted the importance of venom for this role, few species of social wasps have had the potential of their venom investigation. In this sense, the aim of this study was to evaluate the cytotoxicity, mutagenicity and cell viability of *Polybia sericea* (Oliver) venom. Cytotoxicity tests using *Artemia salina*, cell viability test using non-tumor and tumor cell lines and Ames test that assesses genetic and cellular instability were performed. The results show that the venom presents cytotoxicity, with LD_{50} of 22.1 μg mL^{-1}. Mutagenicity was not identified in the Ames test at the concentrations studied. The venom did not present cytotoxicity for MRC-5 cell line, but was cytotoxic for CHO and tumor cell line B16F10-Nex2. Therefore, these tests show that *P. sericea* venom may have potential for pharmacological use, although other studies of cytotoxicity and with more tumor cell lines need to be developed.

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Keywords: ames test; *Artemia salina*; insecta; drug; mutagenicity.

RESUMO

A peçonha de vespas sociais pode ser um produto natural proeminente para o desenvolvimento de fármacos, uma vez que nas últimas décadas estudos têm investigado seu potencial antimicrobiano, anti-inflamatório, antioxidante, anticarcinogênico, entre outros para sua aplicabilidade futura na indústria farmacológica. Embora alguns estudos já tenham destacado a importância da peçonha para este papel, ainda são poucas as espécies de vespas sociais que tiveram o potencial de sua peçonha investigado. Neste sentido, o objetivo deste estudo foi avaliar a citotoxicidade, mutagenicidade e viabilidade celular da peçonha de *Polybia sericea* (Oliver). Foram realizados os testes de citotoxicidade empregando *Artemia salina*, teste de viabilidade celular empregando linhagens não tumorais e tumorais e teste de Ames que avalia a instabilidade genética e celular. Os resultados demonstram que a peçonha apresenta citotoxicidade, com LD_{50} de 22,1 μg mL^{-1}. Não foi identificada mutagenicidade no teste de Ames nas concentrações estudadas. A peçonha não apresentou citotoxicidade para linhagem MRC-5, mas foi citotóxica para a linhagem CHO e tumoral B16F10-Nex2. Portanto, estes testes mostram que a peçonha de *P. sericea* pode ter potencial para uso farmacológico, embora, outros estudos de citotoxicidade e com mais linhagens tumorais precisam ser desenvolvidos.

Palavras-chave: teste de ames; *Artemia salina*; insecta; fármaco; mutagenicidade.

Introduction

Social wasps as well as social bees and all ants are included in the order Hymenoptera, which covers more than 150,000 species (Gulan & Cranston, 2017). These social species have a complex level of behavioral organization to maintain the cohesion of their colonies, such as division of labor between castes and mechanisms of prey capture and colony defense that are extremely efficient (Wilson, 1971).

Among the defense mechanisms, the evolution of a sting apparatus from an ancestral ovipositor stands out, which includes a mechanism of production and injection of venom (Whitman et al., 1990; Brandão, 1999). This structure is generally composed of a secretory part formed by the venom gland and the convoluted gland, in addition to a storage reservoir and a stinger (Cassier et al., 1994).

The venom of these insects acts as a natural weapon to ensure the capture of prey and defense of the colony against predators (Palma, 2013). It is known that the chemical composition of venom includes low molecular mass substances, such as peptides (Mendes et al., 2004; Spillner et al., 2014; Dias et al. 2015), which are often involved in toxic reactions (Arcuri et al., 2016). In addition, natural products are an important source of therapeutic agents (Guo, 2016) and, among these resources, venom may present substances with antitumor properties (King, 2011; Harvey, 2014).

Despite promising research already conducted with social wasps and the fact that 381 species of social wasps occur in Brazil (Somavilla et al., 2021), little is known about the biochemistry, pharmacology, and immunology of their venoms (Santos et al., 2007). Among the obstacles related to the investigation of new bioactive substances from the venom of these insects, the difficulty of obtaining the material in sufficient quantity stands out, since the volume of venom extracted from each individual is reduced, as already pointed out in studies conducted with ants (Fox et al., 2012; Pluzhnikov et al., 2014).

Another challenge is related to the mutagenicity, genotoxicity and cytotoxicity of its substances, which may be a limiting factor for its therapeutic application (Simões et al., 2016). In this respect, studies evaluating the venom regarding its action on genetic instability and cytotoxicity on biological models are relevant for more applied research, such as the study of new therapeutic agents (Freire, 2020).

Despite the difficulties highlighted, some studies have reported efficacy aimed at the application in pharmacology, such as those of Freire (2019), with the venom of *Synoeca* in which an antimicrobial peptide (AMP) classified as mastoparan was reported, and which presents a potent antimicrobial activity and Freire (2020), with the mastoparan peptide of the *Polybia paulista* wasp venom, as a potent trypanocidal agent.

Among the alternative tests for the evaluation of mutagenicity, cytotoxicity, is the Ames test, which is sensitive to mutagenic and cytotoxic agents and which manifest its response based on the induction of reverse mutations using an auxotrophic bacterium for histidine, which in the presence of mutagenic agents reverses its character of auxotrophy and starts to form colonies in an amino acid free medium (Zeiger, 2001; Varella et al., 2004). This *in vitro* test is widely recognized and indicated by government agencies and corporations such as ANVISA, OECD, SBMCTA for evaluation of mutagenic potential, and strains TA 98 and TA 100 are indicated for a *screening*, as they have the ability to detect a large part of the mutagenic agents (Vargas et al., 1993).

In addition, there is the cell viability assay, by the MTT (Thiazolyl Blue Bromide) technique, which is a cytotoxicity assay in which cell viability is measured. This test is based on the ability of viable cells to convert MTT salt into an insoluble purple-colored salt. This product cannot cross the cell membranes of viable cells and therefore accumulates within them (Fotakis & Timbrell, 2006). Current approaches to treatments are often highly invasive, also presenting numerous deleterious side effects. Therefore, anticancer therapic changes are necessary to destroy cancer cells and to prevent toxicity to normal host cells (Torres et al., 2018).

Taking into account that despite the potential of social hymenopteran venoms to be used in an applied way in pharmacology, little is known of their potential biological activities and toxicity, the aim of this study, was to evaluate the cytotoxicity, mutagenicity and cellular viability of the venom of the social wasp *Polybia sericea* (Oliver).

Material and Methods

Collection and preparation of Polybia sericea venom samples

The colonies were collected in the municipality of Dourados-MS (Brazil) (22°11'52.67" S; 54°55'52.64" W) in the state of Mato Grosso do Sul. With the aid of plastic bags and cotton soaked in ether, we enveloped the nest to try to collect as many individuals as possible. Soon after collection, the cotton with ether was removed as soon as possible to avoid any kind of contamination. Then, the wasps were taken to the laboratory, where they were anesthetized under the low temperature action for subsequent extraction of the venom.

The extraction of the venom reservoir occurred by dissection, with the aid of tweezers and a stereoscope microscope the removal of glandular filaments and stinger. To extract the venom, the reservoir was lightly pressed into a 10 ml glass flask, until the release of all its content. Throughout the procedure, all samples were maintained on ice to avoid volatilization and degradation of the substances.

The reservoirs of the extracted venom glands were placed in a 0.5 mL container, containing an aqueous solution of 10% protease inhibitors cocktail. The protease inhibitor is a cocktail of reagents that inhibits the general degradation of proteins in tissues or cell extracts by endogenous proteases, or to investigate particular processes involving blocking the activity of specific proteases (Sigma-Aldrich P2714). After the dissection process, the material was centrifuged at 12,000 RPM for 10 minutes at 4 ºC to separate the membrane of the reservoir from the venom. Then, the venom-containing supernatant was transferred to another microtube, frozen and later lyophilized.

Venom cytotoxicity assay on A. salina

The preparation of the samples and the cytotoxicity test were performed according to the methodology described by Bernardi et al. (2017) modified, and the venom concentrations used in the experiment were: 0.5; 15; 50; 100; 250 and 500 $\mu g\ mL^{-1}$, diluted in saline (pH 8).

The mortality rate at different concentrations was used to construct an analytical curve and then to determine the LD_{50} (Lethal dose for 50% of individuals).

In vitro bioassay to determine the mutagenic and cytotoxic index from the Ames test

The mutagenic potential of venom was evaluated according to the method of Mortelmans & Zeiger (2000). *Salmonella* Typhimurium strains were supplied by the Toxicology and Genotoxicity sector of the Environmental Company of the State of São Paulo.

For the preparation of inoculum, aliquots of 0.1 mL of *S*. Typhimurium TA 98 and TA 100 were inoculated in 20 mL of Nutrient Broth (Oxoid n. 2) and incubated at 37 ºC for 16 h in an incubator with orbital agitation in order to obtain a suspension of approximately $1x10^8$ bacteria mL^{-1}. Then the strains were concentrated by centrifugation (10,000 gyrations at 4°C) for 10 minutes and resuspended in 4 mL of 0.2 mol L^{-1} phosphate buffer.

The assays were performed in the presence and absence of the Microsomal Fraction S9, in order to evaluate the direct and indirect mutagenic potential. 50 µL of phosphate buffer 0.2 mol L^{-1} or S9 fraction, 5 µL of the sample at concentrations: 5, 15, 50, 150 and 500 µg $plate^{-1}$ and 50 µL of bacterial suspension were added to the test tubes. The tubes were pre-incubated for 90 minutes at 37 °C. Then, 2 mL of top agar (0.6% agar, 0.6% NaCl, 0.05 m mol L^{-1} L-histidine, 0.05 m mol L^{-1} biotin, pH 7.4, 45 °C) were added to the tubes and the mixture were poured into plates with minimal agar (1.5% agar, Voguel-Boner 50x solution and 10% glucose solution). The plates were incubated at 37 °C for 48-66 hours and, at the end, the revertant colonies were counted.

The positive controls used in the assays without metabolic activation were: 4-nitro-ophenylenediamine (NPD) (10 µg $plate^{-1}$) for the TA98 and sodium azide (2.5 µg $plate^{-1}$) for the TA100 strain. In the tests with metabolic activation, the same compound 2-antramine (2-ANTR) (2.5 µg $plate^{-1}$) was used for the two strains. Distilled water was used as negative control. All trials were performed in triplicate. The mutagenicity index (MI) was calculated by dividing the number of induced revertants by the number of spontaneous revertants.

Venom was considered mutagenic when the mutagenicity index was equal to or greater than 2 in at least one of the concentrations tested and when there was a dose-response relationship between the concentrations tested and the number of induced revertants (Valente-Campos et al., 2009). Concentrations with MI below 0.6 were considered cytotoxic (Rahdén-Starón et al., 2010).

The results were analyzed by the Statistical Program Salanal (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, EUA, version 1.0, from the Research Triangle Institute, RTP, EUA), adopting the Bernstein et al. (1982) model.

Cellular viability

Cell cultivation

The cell lines used were: MRC-5 (Non-tumor lineage of human fibroblast), CHO (Chinese hamster ovarian non-tumor lineage) and B16F10-Nex2 (tumor lineage of murine melanoma). MRC-5 cells were grown in high glucose DMEM medium. For CHO cells, high glucose and F-10 DMEM medium were used in the 1:1 ratio and for B16F10-Nex2 cells RPMI 1640 medium was used. All cell lines were maintained in laboratory bottles containing their respective culture medium and supplemented with bovine fetal serum 10% added 1% antibiotic (penicillin/streptomycin) at 10 mg mL^{-1}, incubated at 37 ºC and 5% CO_2.

Cell viability test

To evaluate the cytotoxic effect on the viability of non-tumor and tumor cells, a colorimetric assay was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide (MTT). For this, cells were plated in plates of 96 wells and treated with 100µL at varying concentrations. After 24 and 48 h the supernatant was discarded, washed with PBS 1x and added MTT (5 mg mL^{-1} per well). Then, samples were incubated for 4 h in an oven at 37 °C and at the end the supernatant was removed and added 100 µL of DMSO to it. The reading was performed at 570 nm in a microplate reader. The concentrations tested were: 6.25; 12.5; 25; 50; 100; 200 and 400 µg mL^{-1}.

Statistical analyses

For statistical analyses, Two-Way Variance Analyses were used, using statistica 13.3 software (Tibco 2017).

Results

The LD50 of 22.1 µg mL-1 was obtained in the bioassay with *A. salina* for the venom of *P. sericea*. In the Ames test, the concentration of 150 µg $plate^{-1}$ of venom significantly increased ($p < 0.01$) the number of colonies revertants of the TA100 strain of *S.* Typhimurium with metabolic activation (S9+) (Table 1), however the mutagenicity index (MI) was lower than 2 (Table 2). In the other concentrations with metabolic activation (S9+) and without metabolic activation (S9-) the sample did not present mutagenic potential in the strains of *S.* Typhimurium TA 98 and *S.* Typhimurium TA 100 (Table 1). No relationship was observed between concentration and increase of revertants (Table 1). The mutagenicity index was below 0.6 only in the TA 100 strain at concentrations of 150 and 500 µg $plate^{-1}$ (Table 2).

The results that evaluate the effects of venom on cell viability of the MRC-5, CHO and B16F10-Nex2 strains show that its effects do not present significant differences in relation to cytotoxicity for MRC-5 strain at concentrations and times tested ($p< 0.93$, $p< 0.58$) compared to the control group (Fig. 1 A-B). The time also did not affect the other strains tested (Fig. 1 A-B). On the other hand, in the CHO strains the results show that the venom presents significant cytotoxicity in the highest concentrations tested ($p< 0.01$) compared to the control group with dose-dependent effect (Fig 1. A-B).

The results clearly demonstrate that the cells of the B16F10-Nex2 strain were more sensitive to the venom dose-dependent action presenting significant cytotoxicity in all concentrations and times tested ($p< 0.01$, $p < 0.06$) compared to the control group (Fig. 1 A-B). In addition, there is a conclusive reduction in cell viability of more than 60% in the two highest concentrations (200 and 400 µg mL^{-1}).

Discussion

The classification of venom toxicity based on bioassays using *A. salina* allows for an objective evaluation of venom potency across different concentration ranges. According to Sandovai et al. (2020), venom can be considered non-toxic at concentrations above 1000 µg mL^{-1}, low toxic between 500 and 1000 µg mL^{-1}, moderately toxic between 100 and 500 µg mL^{-1}, and highly toxic at concentrations below 100 µg mL^{-1}. Applying this concept, the venom of *P. sericea* show be highly toxic, with LD_{50} of 22,1 µg mL^{-1}. Additionally, Meyer's classification (1982) complements this assessment, suggesting that an LD_{50} below 1000 µg mL^{-1} is indicative of toxicity. Thus, *P. sericea* venom demonstrates high toxicity in the bioassay classification and within Meyer's broader toxicity threshold, highlighting its potent bioactivity.

The venom toxicity test on *A. salina* shows that cytotoxicity is higher than the venoms of some species already studied. For example, Apitoxin of apiary bees presented LD_{50} in *A. salina* of 71.5 μg mL^{-1} and 191.6 μg mL^{-1}, varying depending on the collection site (Abrantes et al., 2017). Indeed, this test has been used to analyze toxins from various animals and insects (Abrantes et al., 2017; Xiao et al., 2018; Hahn & O'Connor, 2000; Okumu et al., 2020; Bickler, 2020).

There is a relationship between the cytotoxicity in *A. salina* of some substances and the cytotoxic effect in cancer cells, which may be indicative of therapeutic potential (Meyer et al., 1982; Moshi et al., 2010; Kumar et al., 2014).

In the Ames test, the modified strains of *S.* Typhimurium in the presence of mutagenic substances alter their auxotrophic character to histidine, and the reading of colonies per plate (Verri et al., 2017) was performed. Through the numbers of the revertant colonies, the mutagenicity index (MI) is calculated, and substances with MI greater than 2 are considered mutagenic (Valente-Campos et al., 2009), while MI below 0.6 are considered cytotoxic (Rahdén-Starón et al., 2010).

TA 98 strain detects frameshift alterations. The results obtained in the Ames test show that *P. sericea* venom did not exhibit an MI greater than 2, a dose-response relationship or statistical significance at any of the concentrations evaluated, with (S9+) or without (S9-) metabolic activation, demonstrating that *P. sericea* venom does not exhibit direct or indirect mutagenic potential under the conditions evaluated (Carneiro et al., 2018).

With regard to the TA 100 strain, it has the ability to detect base pair substitution mutations. It was observed that in the assay with metabolic activation (S9+), although the concentration of 150 μg.$plate^{-1}$ of *P. sericea* venom showed statistical significance, the mutagenicity index was not greater than 2 and there was no dose-response, indicating the absence of mutagenic potential. In the test without exogenous metabolism (S9-), a cytotoxic potential was observed at the highest concentrations of 150 and 500 μg plaque-1, because the MI < 0.6.

Han et al. (2017) and Arani et al. (2019) evaluated the venom of *Apis mellifera* with the Ames test and found antimutagenic and anticancer activity at different concentrations. Varanda et al. (1999) analyzed the venom and propolis of *A. mellifera* bee in the strains of *S.* Typhimurium TA 98 and *S.* Typhimurium TA 100 and observed the absence of mutagenicity in both samples.

The cytotoxic effect on cell viability of the B16F10-Nex2 strain demonstrates a correlation with cytotoxicity on *A. salina*. A reduction in cell viability by an agent can be considered as a cytotoxic effect due to the presence of toxic substances (Zegura et al., 2009). An important fact to be considered in the results obtained is that the venom at the concentration of 100 μg mL^{-1} was highly cytotoxic for tumor cell line B16F10-Nex2, since cell viability above this concentration significantly reduced the cell viability of the tumor cell (Fig. 1 A-B), indicating that *P. sericea* venom presents potential for the treatment of this cancer lineage.

Current drugs have harmful actions on non-target cells, which ends up causing undesirable side effects, and the current approaches to treatments are most often highly invasive, also presenting numerous deleterious side effects. Therefore, anticancer therapic changes are necessary to destroy cancer cells and to prevent toxicity to normal host cells (Torres et al., 2018).

The selectivity observed in the cell line B16F10-Nex2 with no effect on the MRC-5 strain is a promising result, however the effect observed in high concentrations of venom in the CHO lineage may indicate a limitation of direct use and that complementary studies need to be performed.

The most studied social wasp species for the treatment of cancerous tumors is *P. paulista*. The antitumor effects of *P. paulista* venom toxins have already been tested for mastoparan Polybia-MP1 (Wang et al., 2008).

This peptide showed a highly selective cytotoxic activity similar to the results obtained with *P. sericea* venom. According to these same authors, the venom of *P. paulista* presents antiproliferative activity against prostate cancer cells, bladder. Furthermore, Wang et al. (2009) identified the potential of venom of this species for the use of leukemic resistant to multiple drugs.

In other studies, Leite et al. (2015) evaluated that the polybia-MP1 peptide isolated from *P. paulista* venom presents anticarcinogenic activity. While Torres et al. (2018) tested *P. paulista* venom derivatives and observed anticancer activity even at concentrations as low as 12.5 µmol L-1 for selective targeting of MCF-7 breast cancer cells.

The unpublished data obtained in this study indicate that the venom of this species of social wasp presents a biological potential for the treatment of tumors due to the absence of mutagenicity and relative selectivity, opening the possibility for other important aspects to be investigated for pharmaceutical application.

Author Contribution

ERPS: Investigation, methodology and writing – original draft; PFC: Analysis of the Ames test; KMPO: Analysis of the Ames test; DTHC: Analysis of the Cellular viability; ELS: Analysis of the Cellular viability; VOT: Selection and collection of samples and writing – original draft; TLAC: Analysis of the *Artemia salina* and writing – original draft; WFAJ: Investigation, supervision and writing – review & editing; CALC: Conceptualization, investigation, methodology, supervision and writing – review & editing.

Tables

Table 1. Mutagenic activity expressed by the mean of revertants/plate ± standard deviation of *Polybia sericea* venom in relation to the TA 98 and TA 100 strains of *Salmonella* Typhimurium with metabolic activation (S9+) and without metabolic activation (S9-).

	TA 98		TA 100	
Concentrations (µg plate^{-1})	**S9+**	**S9-**	**S9+**	**S9-**
0[a]	13.67 ± 2.52	10.00 ± 0.00	126.67 ± 15.82	150.67 ± 7.09
5	18.00 ± 3.00	17.33 ± 3.51	96.33 ± 1.53	116.00 ± 10.39
15	18.33 ± 3.51	15.67 ± 4.04	90.00 ± 2.65	181.67 ± 13.28
50	18.00 ± 2.65	15.00 ± 3.61	148.67 ± 4.62	103.33 ± 2.31
150	18.67 ± 5.03	7.33 ± 2.08	213.00 ± 6.93**	71.00 ± 3.46
500	9.67 ± 3.21	14.33 ± 5.03	79.00 ± 21.12	18.67 ± 1.15
C+	311.50 ± 4.50[d]	389.5 ± 2.50[b]	307.00 ± 4.24[d]	1619 ± 29.69[c]

0[a]: distilled water used as an extract diluent; Positive Control (C +): [b] 4-nitro-o-phenylenediamine (10 µg plate^{-1}); [c] Sodium Azide (2.5 µg plate^{-1}); [d]2AA-aminoanthracene (2.5 µg plate^{-1}). Significant difference ANOVA: * p<0.05; **p<0.01. Source: Castilho, PF

Table 2. *Polybia sericea* venom mutagenicity index compared to *Salmonella* Typhimurium TA 98 and TA 100 strains with metabolic activation (S9+) and without metabolic activation (S9-).

	TA 98		TA 100	
Concentrations (µg plate^{-1})	**S9+**	**S9-**	**S9+**	**S9-**
5	1.32	1.73	1.31	0.77
15	1.34	1.57	0.71	1.20
50	1.32	1.50	1.17	0.68
150	1.36	0.73	1.68	0.47
500	0.71	1.43	0.62	0.12
C+	22.79	38.95	2.42	10.74

Positive Control (C +): [b] 4-nitro-o-phenylenediamine (10 µg plate^{-1}); [c] Sodium Azide (2,5 µg plate^{-1}); [d]2AA-aminoanthracene (2.5 µg plate^{-1}). Source: Castilho, PF

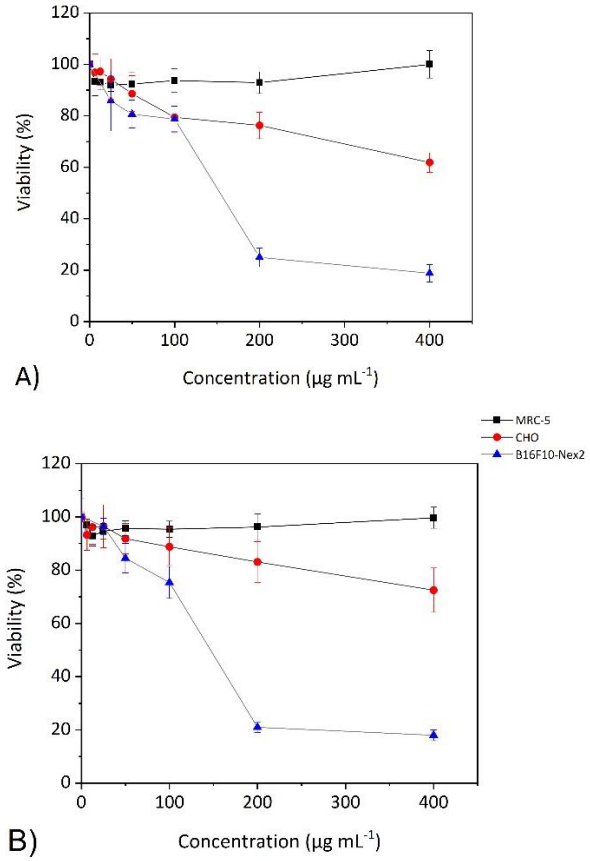

Figure 1 Cell viability by MTT in MRC-5, CHO and B16-F10-NeX2 strain treated with different concentrations of *Polybia sericea* venom. A) Analysis after 24 hours. B) Analysis after 48 hours. Source: Castro, DTH.

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