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The Use of Papain and Benzyl Isothiocyanate as Anthelmintics for Equine Strongyles

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THE USE OF PAPAIN AND BENZYL ISOTHIOCYANATE AS ANTHELMINTICS FOR EQUINE STRONGYLES

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Abstract

Seeds of *Carica papaya* L. (Caricaceae) are a promising source of investigation for a novel anthelmintic to treat equine strongyle infestation. Strongyles are small redworms that attach to the intestinal wall in the equine. They can cause colic, poor hair coat, poor body condition, and, in severe cases, death. The objective of this study was to determine if benzyl isothiocyanate (BITC) and papain, two compounds found in papaya seeds, could be used to prevent migration of third stage cyathostome larvae (L3) *in vitro* using larval migration inhibition assays. The assays involved incubating larvae in solution, followed by larval migration through chambers containing a 25 µm mesh. Larval migration inhibition assays were performed using BITC and papain at concentrations of 0.01 µg/ml, 0.025 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.3 µg/ml, and 0.5 µg/ml. Ivermectin was used as a positive control at 150 µg/ml. Distilled water was used as a negative control. Linear regression was used to compare the responses of larvae to BITC and papain. At the highest BITC concentration (0.5 µg/ml), the percent of larvae that did not migrate (97.1% ± 4.2%), was comparable to the commercially available anthelmintic ivermectin (96.9% ± 4.4%). At the highest papain concentration (0.5 µg/ml), the percent of larvae that did not migrate was 69.4% ± 22.9%. As concentration of each compound increased, so did the number of non-migrating larvae as a percentage of the control. At the concentrations tested, BITC (y=-130.8x+78.5, R^2=0.64, P=0.017) appeared more promising than papain (y= -118.2x+115.4, R^2=0.55, P=0.036). Further testing with additional replicates and more larvae per treatment is needed to confirm these findings.

Introduction

The horse, *Equus caballus*, is very susceptible to gastrointestinal parasites. One of the most prevalent and pathogenic parasites in the equid is the strongyle. Strongyles are parasitic nematodes that are round in body shape and red in color. Sometimes referred to as bloodworms, these parasites have teeth that can latch onto the equine intestinal wall (Nielsen et al., 2007). Strongyles can cause colic, poor hair coat, weight loss, or even death if left untreated (Love et al. 1999). Traditionally, strongyle infestations were treated by deworming with different anthelmintics in a rotation (Swiderski & French 2008). This type of practice basically selects for anthelmintic resistant strongyles. The objective of this study was to determine if benzyl isothiocyanate or papain reduce migration in strongyle populations *in vitro.* To determine if the compounds reduced migration in strongyle populations, larval migration inhibition assays were used testing different concentrations of each compound.

Anthelmintics have been overused and misused in practice by horse owners, leading to issues with parasite resistance to most of these drugs. Ninety-five percent of strongyle populations are resistant to fenbendazole and 40% are resistant to pyrantel pamoate (Coles et al., 2006). Fifty-three percent of strongyle populations are resistant to oxibendazole (Kaplan, 2002). Studies have shown that strongyles are beginning to show early indications of resistance to the effects of ivermectin and moxidectin (Lyons et al., 2008). In Kentucky, 48 horses were evaluated for the presence of strongyle eggs in feces before and after administration of ivermectin. One to two weeks after administration of ivermectin, the feces showed a reduced number of strongyle eggs; however, five to six weeks later strongyle egg numbers increased again (Lyons et al., 2008). Ivermectin was originally effective in suppressing egg counts for nine weeks (Larson et al. 2011).

Ivermectin and moxidectin work by paralyzing the body-wall muscles in nematodes to inhibit their migration within the animal's system. If the nematodes can no longer migrate, they can no longer obtain sustenance, and then they die of starvation (Geary, 2005). Pyrantel pamoate also paralyzes the nematodes, and eventually causes starvation due to lack of motion to attain sustenance. Fenbendazole and oxibendazole bind to a tubulin protein in the nematode and inhibits its ability to metabolize energy, thus paralyzing the parasite. The genetic basis for resistance to the benzimidazoles (fenbendazole and oxibendazole) has been identified. When fenbendazole or oxibendazole are introduced to the parasites, those without the resistant gene, β-tubulin, are killed or hindered (Kaplan, 2002). Those strongyles with the gene will continue to multiply, thus making the gene more and more prevalent and the anthelmintics less effective than before.

Papaya seeds are a promising source of investigation for a novel anthelmintic. Samuels and coauthors (2015) found that ground papaya seeds reduced the number of strongyle eggs shed by horses by up to 95%, with a mean fecal egg count reduction of 69% after two weeks (Samuels et al., 2015). In humans, the crude seeds were crushed and used to treat *Ascaris lumbricoides, Entamoeba histolytica, Necator americanus, Strongyloides stercoralis, Trichuris trichiura, Giardia lamblia, and Taenia saginata* in sixty children in Nigeria (Okeniyi et al., 2007). Fecal samples were taken from each child to determine worm counts before crushed papaya seeds were added to their diets along with honey. The honey was also used alone as a control in another group. The children who had been given both honey and papaya seeds showed a significant reduction of 76.7% for intestinal parasite eggs in stool (Okeniyi et al., 2007). Papaya seeds have also shown efficacy in reducing the number of parasites within humans, sheep, and pigs. Crude papaya seed extract has been tested *in vitro* on earthworms (*Pheretima posthuma*) at a dosage of

60 mg/ml. In this study, they showed efficacy in paralyzing earthworms within 1.88 ± 0.52 minutes and killing the worms within 4.90 ± 0.18 minutes (Sengupta & Banik, 2013). While this showed significant wormicidal ability, more studies are needed on parasitic nematodes to confirm the findings.

Papain, an enzyme found in *Carica papaya* seeds, had a significant effect in decreasing nematode fecal egg counts of sheep, and increasing the lymphocyte numbers, thus showing a beneficial effect on the immune system as well (Ameen et al., 2011). In this study, four groups of 10 sheep were utilized. One group was a negative control and was left untreated; another group was a positive control and was treated with mebendazole, which is a known sheep deworming agent. The other two groups were given an aqueous or a powder form of papain. Papain showed a significant ($P<0.001$) reduction of *Haemonchus contortus, Trichostrongylus, Strongyloides, and Ostertagia* in the fecal egg counts of sheep. It also increased lymphocyte and decreased eosinophil counts (Ameen et al., 2011).

Buttle and coauthors (2015) proved that papain reduced fecal egg counts in sheep upwards of 98% for the parasite *Haemonchus contortus*. The sheep were infected with *Haemonchus contortus* and were given the supernatant of papaya latex. After thirty-five days, fecal counts were taken to determine wormicidal activity. The reduction rate of the parasite eggs was 98%. However, multiple doses did not continue to reduce parasite levels (Buttle et al., 2015)

Benzyl isothiocyanate (BITC) is another compound found in papaya seeds. One study found evidence that BITC can also be used as an anthelmintic to treat human parasites *in vitro* (Kermanshai et al., 2001). Benzyl isothiocyanate is said to be the most potent anthelmintic in papaya seeds due to the fact that it is absorbed in the intestinal epithelium and parasitic worms attach to the intestinal epithelium (Nagesh et al.,2002). Benzyl isothiocyanate was used to treat

the free-living nematode, *Caenorhabditis elegans* (Kermanshai et al., 2001). Treatment with 5 ul BITC/ml solution led to 90% mortality of adult *C. elegans*. However, no further studies in animals or in horses have been conducted.

Crude papaya seeds have been used for centuries in India as an anthelmintic (Kermanshai et al., 2001). Research is necessary to identify the active ingredient in the seeds that can be used to treat strongyle infestations in equids. Benzyl isothiocyanate and papain seem to be the most likely compounds contributing to the efficacy of the crude seeds. Benzyl isothiocyanate works very similarly to ivermectin when tested on muscular tissue in *vitro*. It causes paralysis of the body-wall muscles of nematodes and then death (Wilson et al., 2002). Papain causes damage to the cuticle thus decreasing motility of the nematode and eventually causing death (Page et al., 2014). We hypothesized that BITC and papain would reduce equine strongyle migration *in vitro*.

Materials and Methods

Fecal samples were collected from horses around the area of Columbus, OH. The samples were collected from stall floors within twenty minutes after defecation, and then stored at 4 °C. The samples were processed within one week of collection using a modified Stoll technique. This involved weighing out a sample of feces, adding sucrose flotation solution, centrifuging the samples, and then using microscopy to count strongyle eggs present on the slide. After identifying feces with strongyle egg counts of greater than 1000 EPG (eggs per gram) from only one horse, fresh feces were obtained from that horse and mixed with vermiculite. This product was used to aerate the feces and keep them moist. Feces were placed on cheesecloth over a plastic cup and placed the apparatus in the incubator at 25 °C. The strongyle eggs took fourteen days to incubate and hatch. A Baermann apparatus was used to collect the strongyle larvae from the feces.

The L3 larvae were then pipetted out of the Baermann apparatus, and centrifuged at 1000 x g for 5 minutes. Larvae were exsheathed using a 2% sodium hypochlorite solution made from bleach and distilled water at a 1:4 ratio. After the exposure to the bleach, the exsheathed larvae were washed three times using distilled water and centrifugation at 200 x g for 2 minutes. To ensure the larvae were exsheathed, 25 µl of larval solution was pipetted onto a glass slide and the larvae were checked under a microscope. Exsheathing was performed according to methods of Rendon et al. (2012). The larvae were then Baermannized through a 25 µm mesh for a second time with lukewarm distilled water for two hours at 27°C using a funnel and tubing with a clamp. After the Baermannization, a second larval count was done to make sure that there were at least 35 larvae per 100 µl of solution.

Larvae were then incubated within varying concentrations of BITC or papain in 24 well

microplates (Tables 1 and 2). Distilled water or DMSO were tested in triplicate as a negative control for papain and BITC respectively (Table 2). Ivermectin was also tested in triplicate as a positive control at 150 µg/ml solution (Table 2). Serial dilutions were performed to prepare the working solutions of BITC and papain. Stock solution A was prepared using 10 µl BITC in 11.36 mL DMSO, as BITC is not completely soluble in distilled water. One ml was then taken from stock solution A and added to 9 ml of DMSO to make stock solution B. One ml was then taken from stock solution B and added to 9 ml of distilled water to make stock solution C. Stock solution D was made from 1 ml of stock C and 9 ml of distilled water. These dilutions were done so that the final concentration for DMSO in the working solutions was 0.1%. All working solutions (Table 1) were made from stock solution D. Papain solutions were made in the same fashion as BITC, except that stock A started with 5mg papain and 5 ml distilled water. The rest of the process was the same except distilled water was used instead of DMSO (Table 1). Working solutions (100 µl) were added to 100 µl of larval solution in each well. The working solutions and larval solutions were vortexed for 15 seconds after pipetting into every three wells of the incubation plate. The incubation plate was then incubated in a dark drawer for two hours at 21°C. After the two hour period, the incubation plate was placed on a plate shaker for five minutes at maximum speed.

Next, larval migration inhibition assays were conducted. The larvae were pipetted from each well of the incubation plate into a corresponding well within a new 24-well plate, known as the migration plate. The new plate contained mesh columns and an additional 1800 µl of migration solution containing anthelmintic solutions at differing concentrations (Table 1). The plate was incubated for forty-five minutes at 37°C in total darkness. After the 45 minute period the migration sets were removed and the mesh columns were taken out, turned upside down, and

rinsed with distilled water into another 24-well plate to remove the non-migrating larvae to be counted. For counting of both the migrating larvae and the non-migrating larvae, 50% Lugol's solution was added to each well. The plates were then placed under an inverted microscope at 10x magnification for counting. After counting the final larval numbers per well within the non-migrating plate and the migration plate, the final statistical data were analyzed through linear regression. Results for migrating larvae at each compound concentration were converted to a percentage of the mean negative control migrating larvae. Wells containing fewer than 5 larvae total were excluded from analyses.

Results

Benzyl isothiocyanate was more effective than papain at the concentrations studied (Figures 1 and 2). It was most effective at preventing larval migration at the highest concentration of 0.5 µg BITC/ml. At this concentration, when calculated as a percentage of negative control migrating larvae, 3.0% of larvae were able to migrate. The only concentration that performed similarly was the ivermectin control with 3.2% migrating larvae. Ivermectin was tested at a much higher concentration (150 µg/ml) than the other anthelmintic solutions. At lower concentrations of BITC, there was only a slight effect on strongyle migration. At the lowest BITC concentration of 0.01 µg BITC/ml, 62.2% of larvae migrated. The number of migrating larvae could be predicted by the concentration of BITC within the following formula: -130.8x+78.5, $R^2 = 0.64$ (P=0.017). At differing concentrations of BITC the standard deviation numbers were at varying levels as seen in Table 3.

Papain had an overall percentage of 30.6% migrating larvae at the highest concentration of 0.5 µg/ml (Figure 2). At the two lowest papain concentrations of 0.01 and 0.025 µg/ml, more

larvae migrated than in the control wells. The number of migrating larvae could be predicted by the concentration of papain within the following formula: -118.2x+115.4, $R^2 = 0.55$ (P=0.036). At differing concentrations of papain, the standard deviation numbers were quite different as seen in Table 4.

Discussion

Overall, the results were very promising for the future of finding new anthelmintics for deworming horses. Benzyl isothiocyanate showed more potential for anthelmintic use than papain. The 3% rate of migrating strongyle larvae in 0.5 µg BITC/ml (Figure 1) is very comparable to the 3.2% migrating larvae rate in 150 µg ivermectin/ml. The high efficacy of ivermectin was expected because ivermectin is a known anthelmintic solution commonly used in the equine field; however, ivermectin was also used at a much higher concentration than BITC. In one study by Nagesh and coauthors (2002), 25 ppm of steam distilled BITC had a 100% mortality rate in *M. incogita* and *C. elegans.* The results in our study done at 0.5 µg/ml of BITC or 0.5 ppm were very comparable to this study as there was about a 90% mortality rate of strongyles. The lower mortality rate could be due to not obtaining steam distilled BITC and having a lower level of BITC overall.

The 30.6% migrating larvae in the highest concentration of papain (Figure 2) was still a significant decrease over control results; however, it wasn't as comparable to ivermectin as BITC. Buttle and coauthors (2015) proved that papain reduced fecal egg counts in sheep upwards of 98% for the parasite *Haemonchus contortus* when used at 170 µmol for four days. This data shows that papain may take longer to work at lower levels than BITC.

Both compounds showed high standard deviations for non-migrating larvae per well in some concentrations. This variability is likely due to the lack of numbers in each well and the lack of repeated trials for the experiment due to time constraints. More replications of the experiment would likely remove these high standard deviations and make the data more consistent.

Linear regression analysis results for both compounds were statistically significant, indicating that at higher concentrations they both were more effective than negative controls and have potential for use as equine anthelmintic compounds. R^2 values run between 0% and 100%. The higher the R^2 value, the better the data fits a linear regression plot. Benzyl isothiocyanate R^2 values indicated that 64% of the data fit to a regression line, whereas papain R^2 values indicated that 55% of the data fit to a regression line.

Both of these compounds are found in papaya seeds; therefore, if papaya seeds were used to make a dewormer, there would be anthelmintic effects of both BITC and papain. In developing countries, due to the availability of papaya seeds, the people could effectively use the seeds as anthelmintics for strongyles.

In the future the anthelmintic effects of both BITC and papain will most likely be made into a singular dewormer. Past studies show that equine anthelmintics have a higher rate of efficacy if they contain a least two sources of wormicidal agents. Combined anthelmintic therapy reduces the prevalence and intensity of parasites (Beach et al., 1999). More than likely due to cost factors, BITC and papain will be purchased from a distributor and mixed to make the dewormer instead of using crude seeds.

If this study were to be replicated, more than fifty grams of feces should be used. This way, there would be more strongyles in the overall larval solution. About 10-20% of the overall

larval numbers are lost during the Baermannization process and with exsheathing. These two processes are needed for the migration assays, but if there is a higher overall starting number of larvae then the numbers in the wells won't be as affected. A large number of larvae were also lost during the process of moving solution from the incubation plate to the migration plate. Some of the wells had less than ten larvae overall even with vortexing the larval solution every three wells. This could partly be due to random error caused by pipetting from the same area in the tube every time. However, there are still some discrepancies within the process that make it difficult to get at least thirty-five larvae per well despite the overall initial number of larvae being high enough.

In the future, this experiment should be reproduced with higher numbers of larvae per well and more wells for each concentration. Larvae from different equine centers in Ohio could be used as well to discover if there are different levels of anthelmintic susceptibility in differing larval populations. Eventually, the concentrations that are found to have a greater significance in prohibiting larval migration should be tested *in vivo* to see if the results of the experiment in the lab can be repeated.

Tables and Figures

Table 1. Concentrations of BITC and Papain in working solutions and in wells. The first two columns list the amounts of stock solution D and dH_20 used to make each of the 2x working solutions.

Stock Solution D (µl)	**dH_20 (µl)**	**Papain & BITC concentration in 2x working solution (µg/ml)**	**Final papain and BITC concentration in wells (µg/ml)**	**Incubation solution**	**Migration solution**
108	5292	0.02	0.01	100 µl removed of each working solution and added to 100 µl larval solution in each incubation plate well	900 µl of each working solution was added to 900 µl dH20 to make 1800 µl of each migration solution
270	5130	0.05	0.025		
540l	4860	0.1	0.05		
1080	4320	0.2	0.1		
2160	3420	0.4	0.2		
3420l	2160	0.6	0.3		
5400	0	1.0	0.5		

Table 2. Plate layout (µg compound/ml solution). This layout was used for papain and for BITC

Plate 1	**1**	**2**	**3**	**4**	**5**	**6**
A	(-) control	(-) control	(-)control	0.01	0.01	0.01
B	0.025	0.025	0.025	0.05	0.05	0.05
C	0.1	0.1	0.1	0.2	0.2	0.2
D	0.3	0.3	0.3	0.5	0.5	0.5
Plate 2	1	2	3			
A	(+) control	(+) control	(+) control			

Table 3. Benzyl isothiocyanate concentrations and standard deviations of percent of larvae that did not migrate.

Concentration of BITC	Mean ± standard deviation
0.01	37.8 ± 20.4
0.025	57.1 ± 41.7
0.05	17.5 ± 2.9
0.1	33.3 ± 26.0
0.2	44.4 ± 18.9
0.3	47.9 ± 18.5
0.5	97.1 ± 4.2

Table 4. Papain concentrations and standard deviations of percent of larvae that did not migrate.

Concentrations of Papain	Mean ± standard deviation
0.01	30.5 ± 12.2
0.025	42.1 ± 14.7
0.05	54.8 ± 2.1
0.1	45.5 ± 50.6
0.2	39.4 ± 13.6
0.3	71.4 ± 26.5
0.5	69.4 ± 22.9

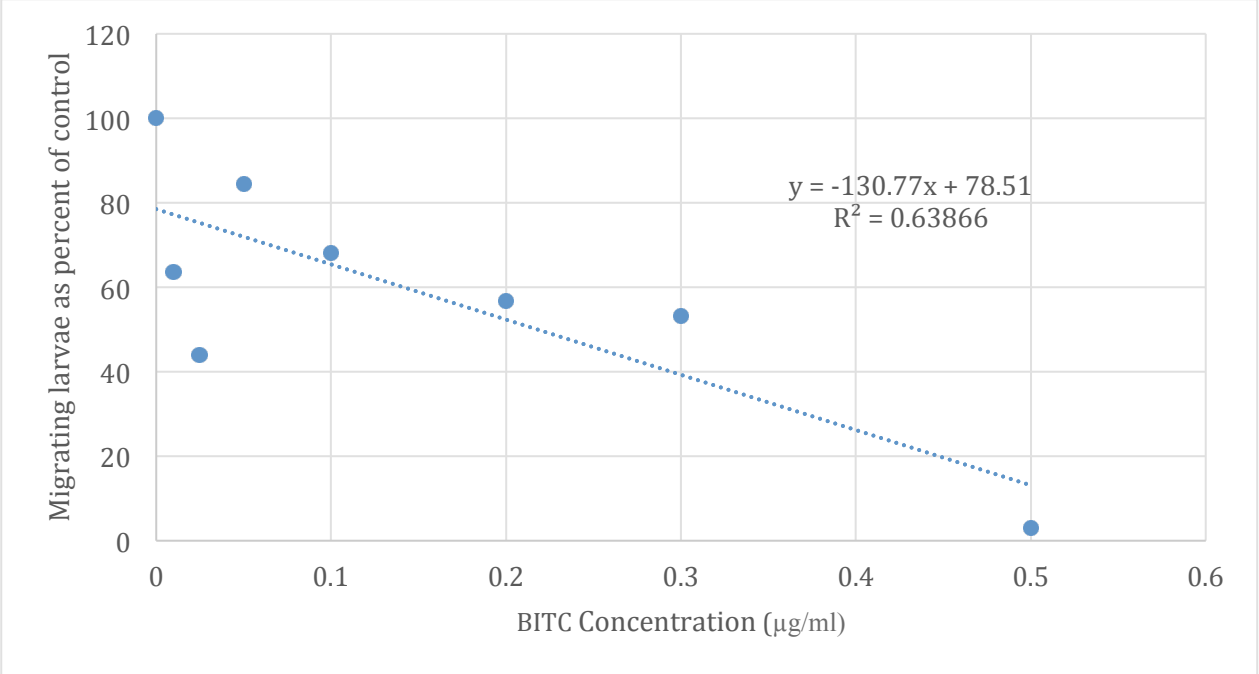

Figure 1. Percentages of migrating larvae at increasing BITC concentrations. Results for migrating larvae at each BITC concentration were converted to a percentage of the mean negative control migrating larvae.

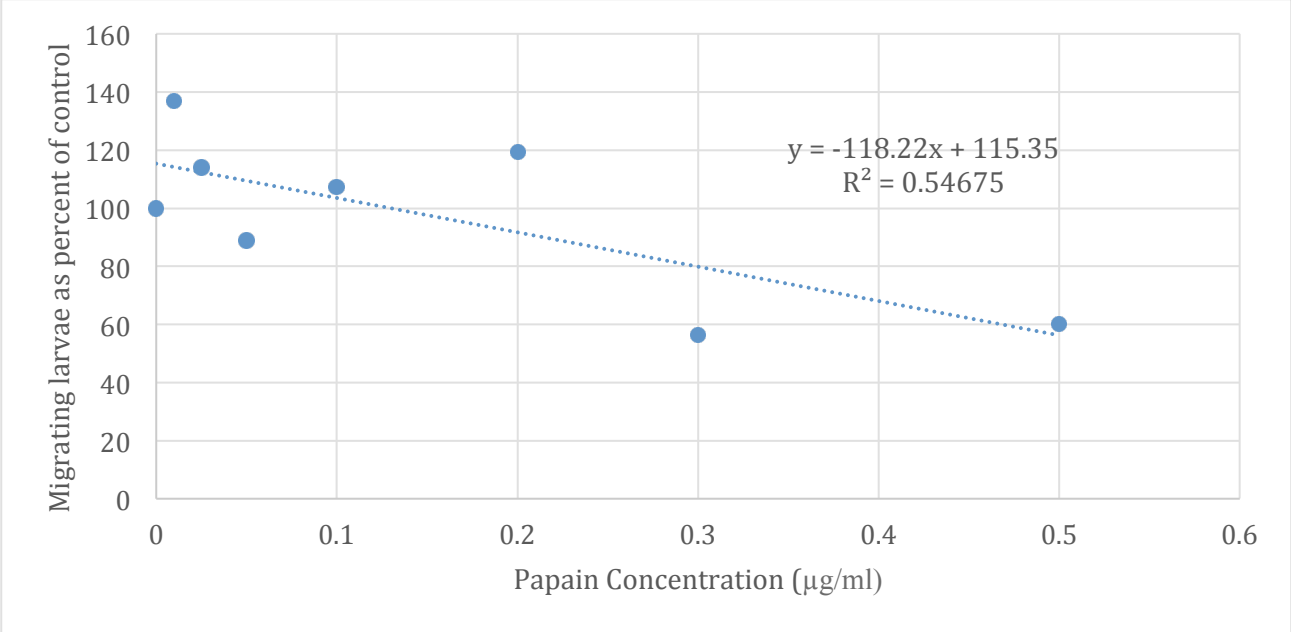

Figure 2: Percentages of migrating larvae at increasing papain concentrations. Results for migrating larvae at each papain concentration were converted to a percentage of the mean negative control migrating larvae.

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