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Preclinical anti-HSV-1 activity of aqueous and methanol extracts of Kenya grown pyrethrum (*Chrysanthemum cinerariaefolium*)

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Due to the opportunistic nature of Herpes simplex viral infections, it is of great public concern in sub-Saharan Africa. To date, there is no vaccine or cure for this viral infection. In this study, anti-viral activity of the Kenyan *Chrysanthemum cinerariaefolium* (Pyrethrum) against Herpes simplex virus (HSV-1) was evaluated *in vivo* (using Swiss mice). Phytochemical screening for presence of secondary metabolites of both methanol and aqueous extract of the plant material (flowers) indicated positive, for presence of alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. The extracts were given orally after acute oral toxicity results (LD50 >2000 mg/kg of body weight) indicated both extracts are safe to be given orally. Upon induction of topical infection with HSV-1 virus, 2 dose levels (10 mg/kg and 25 mg/kg of methanol extract and 25 mg/kg and 50 mg/kg of aqueous extract) of both extracts were administered, 2 times per day for 7 successive days. Results showed Acyclovir (ACV) at 5 mg/kg and organic extract at 10 mg/kg delayed onset of lesion in local regions significantly ($p \leq 0.05$ test vs. control by student *t* test). Also, both the organic extract (at a concentration of 10 mg/kg and 25 mg/kg) and aqueous extract (at a concentration of 50 mg/kg) delayed progression of infection significant ($p \leq 0.05$ test vs. control by repeated measures ANOVA). The results indicate extracts from C. *cinerariaefolium* are active against Hsv-1. So, further investigation is recommended in the Kenya grown *C. cinerariaefolium,* on its bio-active compounds, safety, and activity on other members of the family *Herpesviridae*.

Key words: Anti-Hsv, phytochemical screening, acute toxicity, *Chrysanthemum cinerariaefolium*.

INTRODUCTION

Herpes simplex viruses are DNA viruses. The virus belongs to the subfamily *Alphaherpesvirinae*, in the family *Herpesviridae.* There are two strains of the virus; HSV-1 which is known to cause oral and ocular lesions is a leading cause of viral caused corneal blindness and viral encephalitis (Herpetic Eye Disease Study Group, 1998;

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Shoji et al., 2002). Whereas, HSV-2 is known to cause genital lesions and other serious diseases such as blindness, meningitis, and encephalitis (Connolly et al., 2011), with global estimates of 536 million infected persons with an annual incidence of 23.6 million cases among persons aged 15 to 49 years (Tronstein et al., 2011).

In sub-Saharan Africa, the prevalence in adults ranges from 30 to 80% of females and 10 to 50% of males. A considerable rate of prevalence is indicated in different regions of the world (Anzivino et al., 2009). Acyclovir (ACV) has been used for treatment and prophylaxis of this viral infection (Stranska et al., 2005), but until now, there is no vaccine or cure for HSV infections.

Currently, there is a growing public health concern due to emerging new diseases and the development of resistance by pathogens to the available therapies; such as Acyclovir resistance by HSV strains (Chen et al., 2000**;** Christophers et al., 1998; Coen et al., 1980; Schnipper et al., 1980; Looker et al., 2008). Therefore, there is a need to identify alternative therapies. Pyrethrum plant (*C. cinerariaefolim*) that belongs to the family of perennial plants *Asteraceae*, genus *Chrysanthemum* and species *C. cinerariaefolim* has insecticidal property. Its active insecticidal components are known as Pyrethrins (Morris et al., 2006).

This plant is cultivated widely in Kenya, Australia, Japan, India, New Guinea, Uganda and other countries (Greenhill, 2007). It is used abundantly in many pesticide products in or around buildings (Cox, 2002), crops, ornamental plants, pets and live stocks. Its toxicity is relatively low in humans and other mammals; toxicity in rats is indicated as 4000 times lower than Pyrethrins toxicity to the houseflies (Klaassen et al., 1996).

According to the study by Stanberry et al. (1986), Pyrerhrins exhibited *in vitro* anti HSV activity, the objective of this study was to determine the effect of total extract (methanol and aqueous extracts) derived from the flower of Pyrethrum plant against HSV-1 to identify a new candidate drug. This virus is more virulent and common (Xu et al., 2002; Whitley *et* al., 1998) than HSV-2. Moreover, discoveries from this study would be opening a gate for further research on other members of the family *Herpesviridae*.

MATERIALS AND METHODS

Ethical considerations

The research was conducted in accordance with Kenya Medical Research Institute (KEMRI) guidelines on the international accepted conduct of experimental research and the internationally accepted principles for laboratory research. Approval was obtained from KEMRI Scientific and Ethical Review Unit (SERU) and Animal Care and Use Committee (ACUC) (KEMRI/ACUC/02.07.15).

Plant collection and preparation

Flower heads of *C. cinerariaefolium* were collected from Kiambogo, Nakuru County, Kenya (S 000 41 563', E 0360 25 196') in February 2015. It was air dried at room temperature in a dark room and deposited in the Center for Traditional Medicine and Drug Research (CTMDR) (KEMRI) herbarium with a voucher specimen deposited number Tolo/Mwitari/Keter/001. The plant material was then grinded using laboratory mill (Christy & Norris Ltd., Chelmsford, England) at the Center for Traditional Medicine and Drug research, KEMRI. The plant material was then packed in air tight polyethylene bags to prevent moisture and kept away from direct sunlight until extraction was performed. Percentage yield was calculated as follows.

Extraction

Aqueous extraction

Extraction process was carried out based on slight modification to the method indicated by Awoyinka et al. (2007). Two hundred grams (200 g) of the powdered plant material were soaked in 2000 ml of distilled water and placed in a water bath at 60°C. After 1 h it was decanted into a clean, dry 600 ml conical flask. Filtration was done through 2 layers of sterile gauze. The filtered extract was freeze dried in 200 ml portions using a Freeze Dryer (Edwards freeze dryer Modulyo). Finally, the powder was weighed, labeled and stored in an air tight 50 ml centrifuge tube at 4°C until use.

Methanol extraction

Extraction process was carried out based on a slight modification to the methods used by Parekh et al. (2005). Five hundred grams (500 g) of the dried powder plant material were soaked with 1700 ml methanol in a flat-bottomed 3 L conical flask at room temperature for 3 days in a dark room covered by cotton gauze. After 3 days, it was filtered using sterile cotton gauze and concentrated using a rotary evaporator (Büchi Rota vapor R-114) at 70°C. Finally, the extract was weighed and stored in a cap tight round bottom flack at 4°C until use.

Phytochemical screening

Qualitative analysis in a tube test was carried out to screen for the presence of Alkaloids, Flavonoids, Phenols, Saponins, Tannins and Terpenoids according to the procedures indicated by Wagner (1993), Sofowora (1993), Mace (1963), Kokate (1994) and Segelman et al. (1969), and according the procedures indicated in the *Salkowski test* respectively.

HSV-1 virus

Virus stock was obtained from CTMDR laboratory. It was propagated *in vero* cells (kidney cells of African green monkey) and stored in -80°C freezer until use. Virus titer was determined by end point dilution assay *in vitro*, in Vero cells. Cells were seeded at a density of 1×10^5 cells/well in 96 well plates and were grown in minimum essential media (MEM), at 37 °C under 5 % CO2 incubator for 24 h. After 24 h, the cells were 90% confluent and a virus dilution of 1:10 was prepared in PBS from the original 1 ml virus stock. Then the culture media was replaced with 100 µl PBS containing the virus dilution. The first column contained 1:10 virus dilution and a series of 3 fold dilution was transferred to the subsequent columns. The last 6 columns were used as cell controls. After allowing the virus to adsorb for 2 h, it was replaced with 100 µl maintenance media. Cytopathic effect (CPE) was monitored under light microscope for 2 days and recorded.

Table 1. Design to test efficacy of *C. cinerariaefolium* in HSV-1 infected mice.

Treatment (mg/kg)		Number of mice	Outcome
Placebo	0	8	Not infected/not treated/given water
Control	0	8	Infected /not treated/given PBS with < 10% tween 80
Acyclovir	5	8	Infected/treated
Methanol extract	10	8	Infected/treated
	25	8	
Aqueous extract	25	8	Infected /treated
	50	8	
Total number of animals		56	-

The $TCID_{50}$ (50% Tissue Culture Infectious Dose) was calculated by Spearman and karber algorithm method. A pilot study was done on mice to determine infectious titer; groups of mice were infected with the virus topically starting with highest dilution and infectious titer was chosen rather than the lethal titer.

Acute oral toxicity

The toxicological study was conducted in KEMRI animal house laboratory. It was carried out according to the method described in Organization for Economic Co-operation and Development (OECD) guideline 423 which was adopted in 2001. The experiment was carried out on Swiss mice. Five male and female mice weighing 20 g ± 2 g each were obtained from Kenya Medical Research Institute (KEMRI) animal house and left to acclimatize with the experimental room for 3 days. Each group contained 3 mice from the same sex. The animals were fed with standard feed and water in the course of the study. Both methanol and aqueous extracts of the plant material were administered by oral route in a single dose. The methanol extract was dissolved with Tween 80 < 10% and topped with PBS (Phosphate buffer solution). All the experimental mice received the initial dose of methanol and aqueous extracts at a concentration of 2000 mg/kg of body weight according to the guideline. Two control groups for each extract, a group of one sex were given PBS with < 10% Tween 80. The symptoms and weight were recorded before administration of the extract and during the first 30 min after administration and regularly during the first 24 h after treatment for 14 days daily**.**

Anti-HSV-1 efficacy of *C. cinerariaefolium* in mice

The experiment was performed in KEMRI animal house. A Total of fifty six female Swiss mice (for 2 sets of experiment), aged seven/six weeks from the KEMRI animal house were used. Mice were assigned into 7 groups; each group contained 8 mice. The mice were left to acclimatize to the experimental room housed in a clean shoebox cage, being provided adequate animal feed and clean water. A 2 cm^2 area on each mouse was shaved with an electric hair shaver and the remaining fur on the shaved area was completely removed by a chemical depilatory VEET ® hair removal cream (ingredients declared are; Thioglycolic acid and Potassium hydroxide). Each mouse was assigned randomly into a group. This anti-viral evaluation was done according to the method described by Li et al. (1997).

The shaved area of all mice was wiped with 70% alcohol, and scratched superficially with a bunch of 27 gauge needle to breach the skin slightly to give scarified area of 1cm^2. Then 5 µl suspension of 10^3 tissue culture infectious dose (10^3 $Tcid_{50}$/ml) wild type HSV-1 was applied to the scarified area of the 48 mice. The remaining 8 mice were used as a placebo group; which were not infected nor given any drug or the extract and were only given water. Of the 48 mice 16 mice were used as a control, divided into two groups. The first group, a group of 8 mice, was used as negative control; the negative controls were handled in the same manner as the test mice, but were not treated with the extract or the reference drug ACV and only given PBS with < 10% Tween 80. The second group was given ACV at a concentration of 5 mg/kg. It was used as a reference drug (positive controls) and was given to 8 mice.

Then the remaining 32 mice were divided into 4 groups, each group containing 8 mice. Two groups were given the methanol extract at a concentration of 10 and 25 mg/kg. The other two groups were given the water extract at a dose of 25 and 50 mg/kg of body weight. Experiment design is indicated in Table 1. The extracts and acyclovir were administered orally using oral gavage of 22 gauges (0.72 mm in diameter, 3.8 cm in length), starting 4 h after the initial infection for 7 days 2 times daily. The development of skin lesions was monitored every day for 10 days and mortality was monitored over a period of 30 days. Lesions were scored as follows; 0- no lesion, 2 vesicles in the local region, 4- erosion and/or ulceration in the local region, 6- mild zoster form, 8-zosteriform lesion and 10-serious zoster form lesion and death. The scoring of 0-2-4-6-8-10 is selected to avoid transition points where the score is 0 or 2, 2 or 4, 4 or 6, 6 or 8, 8 or 10. The infected mice were fed and observed for 30 days to determine mortality. In this experiment, the dose levels were chosen based on the cytotoxicity results, which indicated the aqueous extracts to be less toxic.

Data analysis

The student *t* test was used to evaluate the significance of difference between control and treated mice in mean times at which skin lesion was initially scored 2 (vesicles in local region) or 6 (zosteriform lesion) after infection. The repeated measures ANOVA (Benferroni) was used to analyze the interaction between extracts and ACV treatment in mean skin lesion for 3 to 10 days after infection (Li et al., 1997). A p-value ≤ 0.05 was defined as statistically significant. All analysis was done using statistical analysis software SPSS (V. 17)

RESULTS

Percentage yield of aqueous and methanol extracts

The results of the yield of both aqueous and methanol extracts from Pyrethrum flowers are presented in Table 2.

Table 2. Percentage yield of aqueous and methanol extracts.

Medicinal plant	Dry weight (g)	Freeze dry weight (g)	Percentage yield (%)
Aqueous extract	200	13	6.5
-	-	Rotary-evaporated weight (g)	-
Methanol extract	500	65	13

Table 3. Phytochemical screening of aqueous and methanol extract.

Secondary metabolites	Aqueous extract	Methanol extract
Alkaloids	++	+++
Flavonoids	+++	+++
Phenols	+++	+++
Saponins	+++	++
Tannins	+++	+++
Terpenoids	+++	+

+++= deep color, ++= slight deeper, += faint color.

Table 4. Efficacy of aqueous and methanol extracts in mouse model.

Treatment (mg/kg)		Mean days ± S.D		Mortality [B]
		Score 2 [A]	score 6 [A]	
Control	0	3.25±0.35	5.27±0.44	0/8
Acyclovir	5	4.75*± 0.75	7.83±1.51	0/8
Organic extract	10	4.58*±0.57	7.5 ±2.0	0/8
Organic extract	25	3.83±0.83	6.83±1.83	0/8
Water extract	25	4.25±0.5	5.0 ±0.0	0/8
Water extract	50	3.67±0.57	7.5±2.44	0/8

*= $p \leq 0.05$ (test vs. control by student *t* test). A= Mean times at which score 2 or 6 were first observed. B=Number of dead mice against total surviving mice, it was calculated on day 30.

Obtained yield for the aqueous extract was 6.5 and 13% for the methanol extract.

Phytochemical screening of aqueous and methanol extract

Results for the phytochemicals present are represented in Table 3.

Acute oral toxicity results (LD_{50} Determination, OECD 423 guideline)

The results of acute oral toxicity tests are presented in Table 4. Upon receiving both extracts at a concentration of 2000 mg/kg, no mortality was observed. According to the results, the LD_{50} for both extracts in both the sexes is > 2000 mg/kg.

Anti hsv-1 efficacy of aqueous and methanol extracts in mice model

After the initial infection, onset of the infection was observed as development of vesicles in a local region (score 2) on Day 3 for the controls and Day 4 for those given the ACV 5 mg/kg. For the test groups, vesicles on the local region (score 2) was observed on days 4 and 3 for those given the organic extract at a concentration of 10 and 25 mg/kg, respectively. For those mice that were given the aqueous extract, vesicles were observed on days 4 and 3 at a concentration of 25 and 50 mg/kg, respectively.

Mild zosteriform lesions (score 6) were observed on Day 5 and 7 for the controls and for those treated with ACV at 5 mg/kg, respectively, for the test groups, mild zosteriform lesions (score 6) were observed on Days 7 and 6 for those given organic extract at a concentration of 10 and 25 mg/kg, respectively. For the groups which were

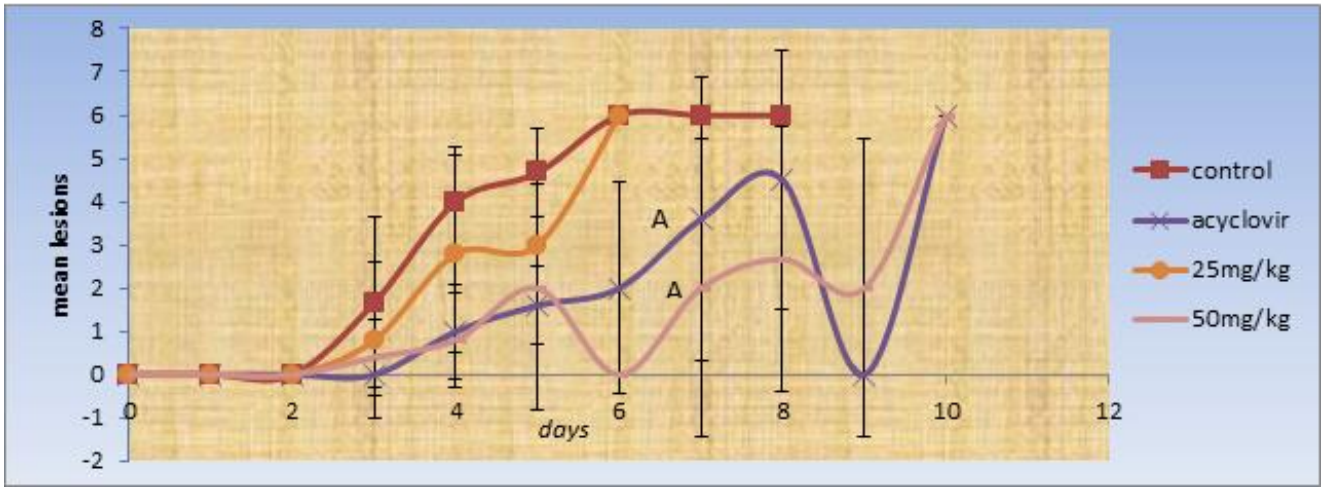

Figure 1. Interaction line plots for progression of HSV-1 infection in Swiss albino mice following oral treatments with aqueous extract of *Chrysanthemum Cinerariaefolium* (10 mg/kg, 25 mg/kg) and Acyclovir 5 m/kg, $p \leq 0.05$ Test Vs control by repeated measures ANOVA (Benferroni). Error bars are deviations within the mean of 8 mice in each group. A= significant.

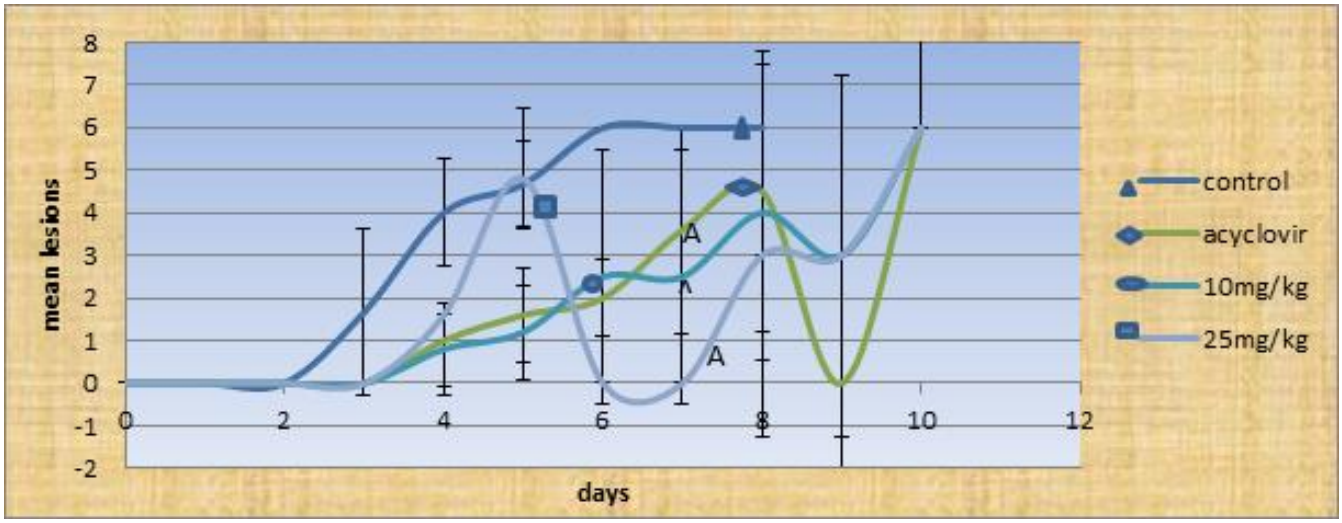

Figure 2. Interaction line plots for progression of HSV-1 infection in Swiss albino mice following oral treatments with methanol extract of *Chrysanthemum Cinerariaefolium* (10 mg/kg, 25 mg/kg) and Acyclovir 5 m/kg, ($p \leq 0.05$ Test Vs. control by repeated measures ANOVA (Benferroni). Error bars are deviations within the mean of 8 mice in each group. A= Significant..

were given the aqueous extract at a concentration of 25 mg/kg mild zosteriform lesions was observed in Day 5 and for those which were given 50 mg/kg mild zosteriform lesions was observed on day 7. In both experiments there was no progression to a Sever mild zosteriform lesion and no mortality was observed up to the 30th day.

Figure 1 displays the interaction of the line plots for progression of HSV-1 infection in mice, following oral treatments with aqueous extract at 25 and 50 mg/kg. The progression of infection in mice treated with 25 mg/kg was similar to that of control. The 50 mg/kg treatment kept the progression of infection the same as those which were given Acv at 5mg/kg and the influence was significant ($p \leq 0.05$ test Vs control by repeated measures ANOVA (Benferroni), but when the treatment was stopped at the 7th day there was an upsurge in the progression of the infection. Figure 2 displays the interaction of the line plots for progression of HSV -1 infection in mice. Following oral treatment with the methanol extract at a concentration of 10 and 25 mg/kg, there was significant delay ($P \leq 0.05$) in the progression of the infection in comparison to the control. By repeated measures ANOVA (Benferroni) as does the acyclovir at 5 mg/kg. But upon withdrawal on the 7th day infection

progressed.

DISCUSION

C. cinerariaefolim (Pyrethrum) plants are well known for their insecticidal properties. It contains active insecticidal compound known as Pyrethrins (Morris et al., 2006). There are different varieties in the world, in this study the Kenya grown variety was tested for its anti-viral activity against HSV-1 in mice. According to previous studies (USEPA, 1994), Pyrethrins which are extracted from this plant showed no observable adverse effects at a concentration of 686 and 834 mg/kg in both male and female mice, respectively.

In this study, mice were given orally the total extract of both aqueous and methanol at a concentration of 2000 mg/kg, and no mortality was observed. Increase in heart beat in some mice was noticed during the first 20 min. According to a previous study (Rao et al., 1973; Sashida et al., 1983) flavonoid, fatty acids (Head, 1968) and essential oils (Saggar et al., 1997) were identified in Pyrethrum plant.

In this study aqueous and methanol extracts of Pyrethrum were screened for the presence of secondary metabolites even though the technique used in this experiment is qualitative analysis, both extracts were tested positive for the presence of secondary metabolites: alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. These secondary metabolites from other plants have shown bioactivity, as it is indicated elsewhere were flavonoids (Pengsuparp et al., 1995) and alkaloids (McMahon et al., 1995) contain anti-HSV activity.

According to the study done by Sassi et al. (2008), *C. trifurcatum* grown elsewhere exhibited *in vitro* anti-HSV activity, also, Stanberry et al. (1986) indicated, Pyrerhrins the active insecticide from C. *cinerariaefolim* exhibited *in vitro* anti HSV activity. In this study also both extracts when tested for their effect on HSV-1 *in vivo* in mouse model at different concentrations, showed an activity that might be attributed to the presence of the secondary metabolites, the methanol extract at 10 mg/kg delayed onset of infection significantly, as does the reference drug (ACV 5 mg/kg). Both dose levels of the methanol extract delayed progression of infection significantly as does the reference drug, and kept the infection to its minimum level, but upon withdrawal of the extract on the 7th day there was progression of infection unlike the reference drug which was seen to contain the infection. The Aqueous extract at 50 mg/kg was also observed to contain progression of infection significantly, but upon withdrawal of the extract on the 7^{th} day the infection progressed. For both extracts, the initial onset of lesions were seen to be delayed at the lower concentration, while there was a delay on the initial score of zoster formation at the higher concentration for both extracts. The reason for this cannot be predicted in this study. In this experiment no mortality was observed in all the groups, this is because the infectious titer of the virus was chosen rather than the lethal titer.

Conclusion

In this study, *C. cinerariaefolim* tested positive for the presence of secondary metabolites. It also demonstrated a significant anti HSV -1 activity. It is therefore recommended that, further study is required for its mode of action. Also, this plant extract should be tested on those emerging Acyclovir resistant strains and on other members of the family *Herpesviridae* which are also concern for a public health.

Conflict of Interests

The authors have not declared any conflict of interests.

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