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Phytochemical, Antioxidant and *In Vitro* Wound Healing Potential of Ethanolic Leaf Extract of *Ruellia tuberosa*

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A B S T R A C T

The objective of this study was to examine the in vitro wound healing effects of the ethanolic extracts of *Ruellia tuberosa* obtained by cytotoxicity, cell scratch assay, antioxidant activity, and selected phytochemical constituents. Qualitative phytochemical analysis and quantitative phytochemical analysis of Total phenolic and flavonoid contents were measured using spectrophotometry methods. The cytotoxic effects of the extracts on 3T3 cell line were evaluated by Mosmann, (1983) method. Furthermore, migration and spreading of the treated fibroblast cells were assessed by cell scratch assay as an *in vitro* wound healing model. The results of the cytotoxicity assay indicated that the ethanolic extract did not have any cytotoxic effect on fibroblast cells The cell was exposed to different concentrations of test compound for 48hrs and the cytotoxic effect of the extract was evaluated. The percentage viability 3T3 Cell line at the highest treated concentration of *Ruellia tuberosa* leaf extract was observed to be 77.97. Fibroblast migration was significantly increased by sample treatment compared to the control. The extracts showed good antioxidant activity and phytochemical compounds. The results showed that *Ruellia tuberosa* leaf extracts have wound healing potential and contain several important antioxidant phenolic compounds. This species deserves further investigation aiming to isolate and identify the active compounds.

***Key words*:** Wound healing, *Ruellia tuberosa,* Ethanolic extract, Antioxidant

Modern assessments show that around 6 million people suffer from chronic wounds worldwide. Wounds related to diabetes, gastric disorders, and duodenal ulcers and due to injuries, such as cuts and burns continue to have serious impacts on the life quality of patients [1]. The term wound generally refers to the disruption in the normal architecture of skin, which form the outer protective layer and the largest organ of the integumentary system for all animals and human beings. The skin plays a critical role in fluid homeostasis and provides sensory functions and thermal regulation. About 15-25% of the total body weight composed of skin, and it receives approximately one-third of body's blood supply at the rate of 300ml/minute. Three main factors can cause injuries to skin and thereby leading to wounds. These factors include environmental, mechanical and chemical. The environmental factors include wind, temperature irregularities, humidity and sunlight. Mechanical injuries may result due to friction, shear force, pressure and epidermal stripping. The chemical injuries are caused by certain irritant chemical substances like corrosive acids, phenol, etc. [2].

Plants are a good source of chemically diverse phytoconstituents and these are assimilated by human and animal bodies easily as compared to the synthetic molecules and hence render a pharmacophore for the development of drugs. Several papers have been published indicating the investigation of plant extracts for their potential as wound healing agents, however very few plants have been investigated in detail explain the constituents responsible and the mechanism of action of wound healing. Chemically phyto-constituents are classified into major categories like alkaloids, glycosides, terepenoid, quinines, flavonoids, polyphenols, sulphur-containing compounds, polyacetylenes, polyketides and steroids. It is observed that the compounds having anti-inflammatory, antibacterial, astringent, antioxidant activities and immunomodulatory activities indicated to promote the wound healing process [3].

The objective of this study was to assess the wound healing potential of *Ruellia tuberosa* ethanolic extracts by *in vitro* methods, as well as to investigate their cytotoxicities, antioxidant activities, and phytochemical compositions with special emphasis on phenolic compounds. Moreover, we compared ethanolic extract of leaf and stem extracts obtained by maceration and Soxhlet methods.

MATERIALS AND METHODS

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Collection of plant material

The leaf and stem of Ruellia tuberosa were collected from nearby villages of Tirupattur district, Tamil Nadu and the leaves are washed with water and dried carefully in the absence of sunlight to remove the water molecules present in the leaves. The dried leaves and stem are made into fine powder using blender. Then the fine powders are stored properly in an airtight container for future purpose.

Extraction of plant material

About 40gm of the fine powder of the leaf and stems of Ruellia tuberosa are taken in a thimble which is placed in a Soxhlet extractor for the purpose of extraction of phytochemicals present in the leaves. The extraction is carried out using ethanol. The extracts obtained are collected separately and the solvents are evaporated using vacuum distillation and dried. The dried samples are stored in an airtight container for further analysis.

Qualitative phytochemical screening

Phytochemical screening

The qualitative tests were carried out in leaf and stems of *Ruellia tuberosa* by adopting standard procedure [4-6]. The ethanolic extract were screened for the presence of phytochemicals.

1. *Test for alkaloids*:

Small portion of solvent free extract was stirred with few drops of dil HCl and filtered. The filtrate was then tested for following colour test:

Mayer's test:

(a) 1.36 gm of mercuric chloride was dissolved in 60 ml distilled water.

(b) 5gms of potassium iodide was dissolved in 20 ml of distilled water (a) and (b) was mixed and the volume adjusted to 100ml with distilled water. Appearance of cream colour precipitate with Mayer's reagents showed the presence of alkaloids.

2. *Test for flavonoids*

Shinoda's test: 5 ml of 20% sodium hydroxide was added to equal volume of the extract. A yellow solution indicates the presence of flavonoids.

3. *Test for steroids*

Liebermann Buchard test: A small amount of sample is treated with 2ml of acetic an-hydride followed by the addition of 3ml of H_2SO_4 solution. Color changes from violet to green or blue indicates the presence of steroids.

4. *Test for terpenoids*

Salkowski test: To 1ml of extract add 0.5ml of chloroform followed by a few drops of concentrated sulphuric acid, formation of reddish-brown precipitate indicates the presence of terpenoids.

5. *Saponins*

Froth test: 5ml of extract is diluted with 20ml of distilled water and agitated for 10 minutes. Foam is formed which indicates the presence of saponins.

6. *Test for carbohydrates*

Fehling test: Two milliliters of each plant extract were hydrolyzed with dilute HCl, neutralized with alkali, and then heated with Fehling's solution A and B. The formation of a red precipitate was an indication for the presence of a reducing sugar.

7. *Test for tannins and phenolic compounds*

Lead acetate test: 10% lead acetate solution, 0.5g of the extract was added and shaken to dissolved. A white precipitate observed indicate the presence of tannins and phenolic compounds.

8. *Test for glycosides*

Keller-Killani test: To 2ml of extract, glacial acid, one drop 5% ferric chloride and concentrated sulphuric acid were added. Appearance of reddish-brown color at the junction of the two liquid layers indicates the presence of glycosides.

9. *Test for quinones*

Sulfuric acid test: One drop of concentrated sulfuric acid was added to 5 ml of each extract dissolved in isopropyl alcohol. Formation of red color indicates the presence of quinones.

Quantitative phytochemical analysis

Estimation of flavonoids

The total flavonoid content in the sample was estimated by the method of Chang *et al*. [7]. The extract prepared for the estimation of total phenolics was used as sample for this assay. A volume of 0.25 ml of the sample was diluted to 1.25 ml with distilled water. A volume of 75 µl of 5% sodium nitrite was added and after six minutes 0.15 ml of aluminium chloride solution was added. A volume of 0.5 ml of 0.1M NaOH was added after 5 min and made up to 2.5 ml with distilled water. The solution was mixed well and the absorbance was read at 510 nm in comparison with standard quercetin at 5-25 µg concentration. The results are expressed as mg of flavonoids as quercetin equivalent / gm of dried sample.

= Test optical density / Standard optical density × Standard concentration

= ---------- / S1 Standard volume × 100

= ---------- µg / 1000

= ---------- mg of total flavonoid content

Determination of total phenols

Total Phenolic extract was assessed using Peri and Pompei [8]. Five ml of the extract was pipetted into a 50 ml flask, then 10ml of distilled water was added. A volume of 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for color development. This was measured at 505 nm.

= Test optical density/Standard optical density ×standard concentration

= ---------- / S1 Standard volume × 100

= ---------- µg / 1000

= ---------- mg of total phenolic content

Determination of tannin

The tannin content in the sample was estimated by the method of Peri and Pompei [8]. 1 mL of saturated sodium carbonate solution were added to 0.5 mL Folin- Denis reagent. The volume was made up to 10 mL with distilled water. After 30 min the tannins content was measured at 760 nm with the spectrophotometer against experimental blank adjusted to zero absorbance. Tannic acid was used as a standard compound.

= Test optical density/Standard optical density ×standard concentration

= ---------- / S1 Standard volume × 100

= ---------- µg / 1000
= ---------- mg of tannin content

2,2-Diphenyl-1-Picrylhydrazyl free radical scavenging activity assay by Brand-Williams *et al.* [9].

The extracts were prepared in concentrations of 10, 20, 30, 40, and 50 µg/mL for this assay. First, 3 mL of extract of each concentration was mixed with 1 mL of the 0.1 mmol/L DPPH solution prepared in methanol. Next, the tubes were incubated in the dark at room temperature for 30 min and then read at 517 nm using a UV-VIS spectrophotometer. Solvent without extract was used as a negative control and AA was used as a positive control. The effect of antioxidant capacity was observed as the colour change of purple DPPH to yellow/light-yellow and % inhibition values of each extract were calculated using the following equation:

Inhibition (%) = [(Acontrol — Ablank) — (Asample — Ablank)] × 100 / (Acontrol — Ablank),

Where A control is the absorbance of the negative control and A sample is the absorbance of AA or extracts. Inhibitory concentration (IC50) values were calculated with inhibition rates using a four-parameter logistic regression model after sigmoidal curves were plotted. Each of the standards and the samples were measured in triplicate and mean values were used for the calculations.

Cell line and culture

3T3 L1 cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37°C.

In vitro assay for cytotoxicity activity: (MTT assay) by [10]

Cells (1 × 105/well) were plated in 24-well plates and incubated in 370C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 hours. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Cell scratch wound healing assay

The 3T3 cell line was used for wound healing assay. The cells were seeded into the 6-well plate and incubated for 24 hours. After incubation, the cells were observed for growth and assay was preceded. The medium was discarded and the plate was kept under microscope. A sterile tip was used and wound was created and wells were washed with sterile PBS in order to wash the detached cells 1 ml of the sample of desired concentration was added to the well and incubated. Control well (without the sample) was also maintained. After 24 hours of incubation, the plate was observed for the growth of cells. The migration capabilities of 3T3 L1 cell line were assessed using a cell scratch in vitro wound healing assay, which measures the expansion of a cell population on surfaces. Solvent without the extract was added to the negative control wells. The cells were visualized under an inverted microscope. The area between the scratch edges was calculated by image processing using imageJ software. Firstly, the edges of the cells were contoured and then the cell-free area in between was calculated based on pixels. The closure rate was calculated with these values using the following formula:

Closure rate = [(Areat0 — Areat24)/Areat0] × 100

Where Areat0 is the calculated area value at 0 h and Areat24 is the area value at 24 h.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis of leaf and stems extract

Qualitative phytochemical analyses were performed for ethanolic extracts of *Ruellia tuberosa* in order to detect the presence of multiple phytochemicals viz, alkaloids, carbohydrates, flavonoids, glycosides, phenol, quinones, steroids, saponins, tannins and terpenoids. Results are shown in the (Table 1). Glycosides, quinones and saponins are nonappearance in ethanolic leaf extract and in stem extract, Glycosides, quinones and saponins and terepenoids are not present. Based on the finding in both leaf and stem ethanolic extract of the study plant, ethanolic leaf extract contain more phytochemicals.

Table 1 Phytochemical screening from leaf and stem ethanolic extract of *Ruellia tuberosa* L.

Phytochemicals	*Ruellia tuberosa* leaf extracts	*Ruellia tuberosa* stem extracts
Alkaloids	+	+
Carbohydrates	+	+
Flavonoids	+	+
Glycosides	-	-
Phenols	+	+
Quinones	-	-
Steroids	+	+
Saponins	-	-
Tannins	+	+
Terpenoids	+	-

(+) indicates positive and (-) indicates negative

Quantitative phytochemical analysis

Quantitative analysis on the basis of results obtained by qualitative test of *Ruellia tuberosa* quantitative analysis of the phytochemicals were performed by using standard method for major phytochemicals such as phenolic content, flavonoids and tannins. Quantitative analysis depicted the higher concentration of phenolic content, flavonoid and tannin in leaf extract compared to stem extract (Table 2).

Table 2 quantitative phytochemical analysis

Phytochemicals	Ethanolic extract of leaf	Ethanolic extract of stem
Total phenolic content	0.26±0.01	0.08±0.05
Flavonoids	0.33±0.05	0.23±0.01
Tannins	0.43±0.01	0.28±0.01

Mean ± Standard deviation (n=3) (p≤0.005%)

Antioxidant activity

The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of plant extract. The IC_{50} value of ethanolic leaf extracts of study plant is 43.76 µg/ml and ethanolic stem extracts of study plant is 46.34µg. The standard ascorbic acid IC50 value is 40.4µg. The result of IC50 value showed that ethanolic extract of leaf is exhibited highest antioxidant activity compare to the stem extract of *Ruellia tuberosa* L. (Fig 1).

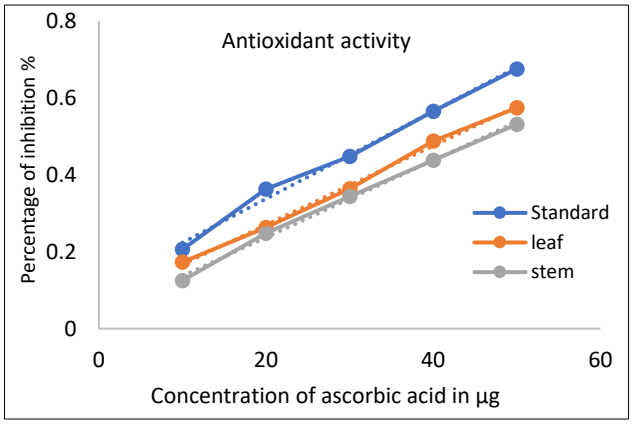

Fig 1 Antioxidant activity of *Ruellia tuberosa* leaf and stem extract

Cytotoxicity effect of Ruellia tuberosa leaf extract

While plant extracts have been extensive studied for their medicinal properties, the cytotoxic effects of such extracts on the cell type of interest are sometimes ignored. However, in the recent times, there has been a growing trend in testing this critical component [11-12]. Cytotoxic effect of *Ruellia tuberosa* leaf extract on 3T3 Cell line was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell was exposed to different concentrations of test compound for 48hrs and the cytotoxic effect of the extract was evaluated. The percentage viability 3T3 Cell line at the highest treated concentration of *Ruellia tuberosa* leaf extract was observed to be 77.97. The concentrations of *Ruellia tuberosa* leaf extract used for treatment and their corresponding percentage cell viability were tabulated in (Table 3) and represented in (Fig 2-3). These effects indicated that the extract was not cytotoxic and could be assessed for their medicinal assets.

Table 3 Cytotoxicity effect of sample on 3T3 L1 cell line

Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1000	Neat	0.393	77.97
500	1:1	0.401	79.56
250	1:2	0.412	81.74
125	1:4	0.420	83.33
62.5	1:8	0.429	85.11
31.2	1:16	0.436	86.50
15.6	1:32	0.445	88.29
7.8	1:64	0.456	90.47
Cell control	-	0.504	100

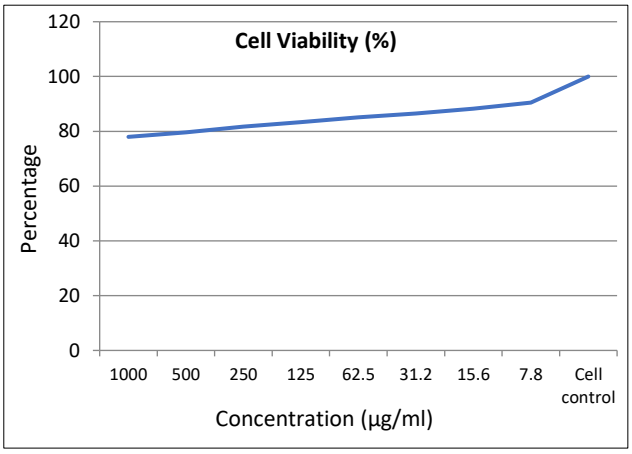

Fig 2 Cytotoxicity effect of sample on 3T3 L1 cell line

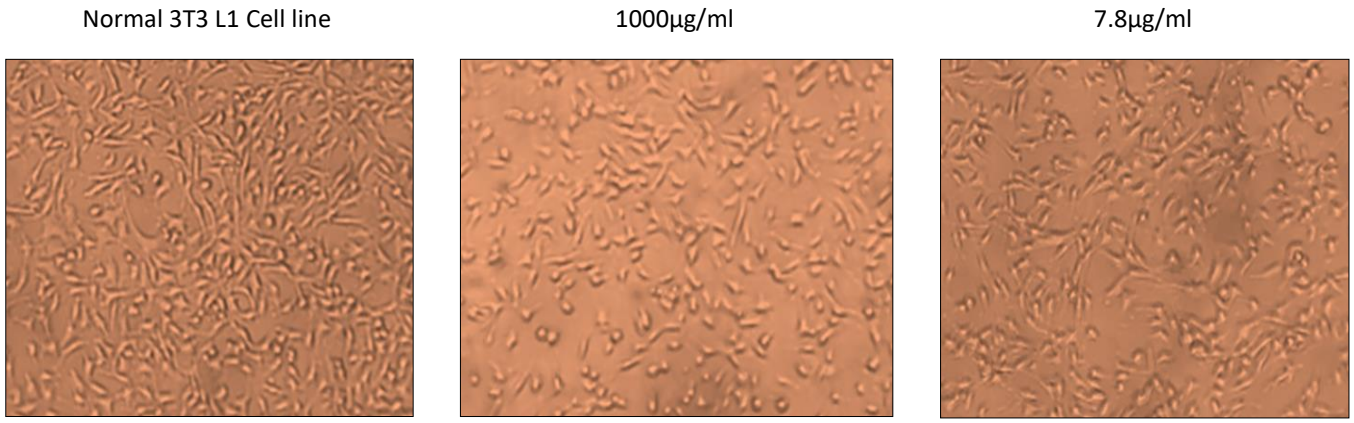

Fig 3 Cytotoxicity effect of sample on 3T3 L1 cell line

Fibroblast cell migration was induced by Ruellia tuberosa leaf extract

Activation, proliferation and migration of fibroblasts are the primary steps in wound healing, where multiple cell types and other micro environmental factors are involved. Scratch assay is a widely applied *In vitro* technique for understanding the wound healing capabilities of medicinally important compounds [13]. In the current study, 3T3 Cell line was treated with 100 µg/mL of *Ruellia tuberosa* extract for 24 hrs. Cell migration at Zero hour and 24 hours were captured and wound closure distance was calculated. The results indicated that *Ruellia tuberosa* leaf extract, at 100 µg/mL, closed the gap created by the scratch by 72.28% in 24 h. *Ruellia tuberosa* leaf extract induced the migration of 3T3 Cell line resulting in wound closure. In the control treated cells, 63.85% of the gap was closed at 24 hr. (Fig 5) shows the microscopic pictures of wound created, control and extract-treated 3T3 Cell line. The pictures demonstrate increased cell migration in the control drug-treated cells and extract treated cells and wound closure percentage was given in (Fig 4).

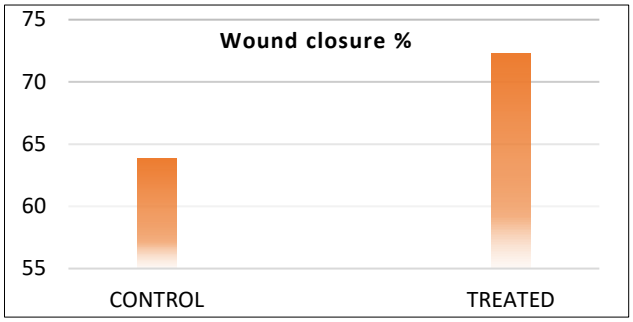

Fig 4 Wound closure percentage of *Ruellia tuberosa*

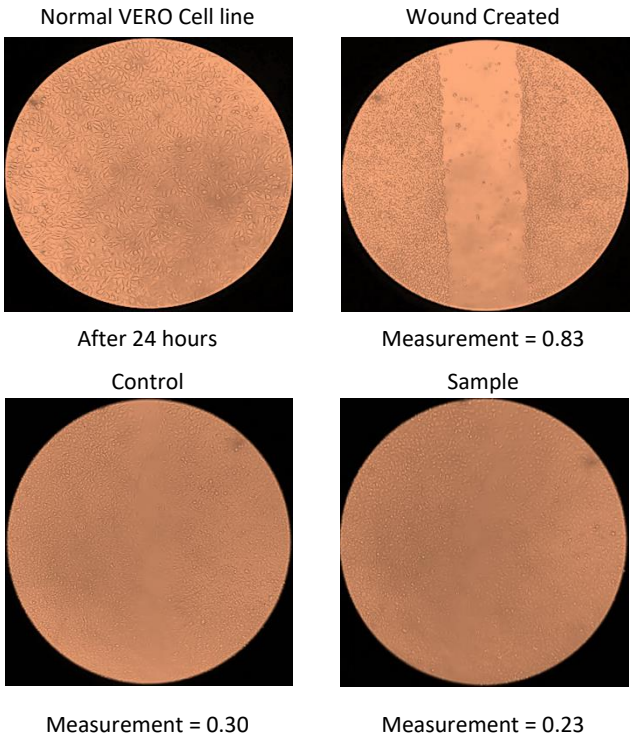

Fig 5 Microscopical images representing the *in vitro* wound healing nature of ethanolic extract of *Ruellia tuberosa*

3T3 Cell line were incubated in presence or absence of *Ruellia tuberosa* leaf extract and control, images were captured at after 24 hours. (a) Normal, (b) Wound created (c) 100 µg/ml of *Ruellia tuberosa* leaf extract. The boundaries of the scratched wounds were determined by the dark lines.

After 24 hours

$$\text{Wound Closure \%} = \frac{\text{Measurement at 0th hr} - \text{Measurement at 24th hr}}{\text{Measurement at 0th hr}} \times 100$$

$$\text{For Control} = \frac{0.83 - 0.30}{0.83} \times 100 \Rightarrow 63.85\%$$

$$\text{For Sample} = \frac{0.83 - 0.23}{0.83} \times 100 \Rightarrow 72.28\%$$

CONCLUSION

Wound healing is a complex and active process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contraction is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. The wound-healing property of *Ruellia tuberosa* may be attributed to the phytoconstituents present in the plant, and the quicker process of wound healing could be a function of either the individual or the additive effects of the phytoconstituents. The present study revealed that the ethanolic leaf extract was found to possess significant wound healing potential.

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