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# EVALUATION OF *TRIDAX PROCUMBENS* LEAF EXTRACT LOADED PVA FILM FOR WOUND HEALING APPLICATION

Harsini Venkatachalam and Radha Palaniswamy *

Department of Biotechnology, Dr. NGP Arts & Science College, Dr. NGP Nagar, Kalapatti road, Coimbatore - 641048, Tamil Nadu, India.

**Keywords:**

*Tridax procumbens*, PVA, Compatibility, Wound healing, Hemolytic assay

**Correspondence to Author:**
**Dr. Radha Palaniswamy**

Assistant Professor,
Department of Biotechnology,
Dr.NGP Arts & Science College,
Dr.NGP Nagar, Kalapatti road,
Coimbatore - 641048, Tamil Nadu,
India.

**E-mail:** palaniswamyradha@gmail.com

**ABSTRACT:** Wounds are a severe health problem. The development of pharmaceutical dosage forms to ensure patient comfort and safety and optimize treatment effectiveness is of great interest to the pharmaceutical and biomaterials industries. *Tridax procumbens* (*T. procumbens*) leaf is a medicinal herb used for treating wounds since ancient times. In this work, a biomaterial film was developed by using PVA with T. procumbens leaf extract via solvent casting method to produce effective wound healing material. According to the hemolytic investigation, films made from T. procumbens leaf extract films were biocompatible. Using L929 mouse fibroblast cells, the cytotoxicity of films was examined using the MTT assay, and the same cell line was also used for a migration assay using the scratch test. The results of the *in-vitro* wound healing test showed that films containing 12.5 ml *T. procumbens* dried leaf extract are efficient in reducing the scratch open area, provoking nearly complete closure of the scratches (94%) within 24 hr without cytotoxicity. The 12.5 ml incorporated film demonstrated better wound healing potential than the 25 ml and 50 ml extract-incorporated films in fresh and dried *T. procumbens* leaf extract-incorporated films. The L929 mouse fibroblast cell's increased vitality and motility indicate that the synthesized film has potential as a wound healing application.

**INTRODUCTION:** Skin primarily acts as a barrier against pathogens and excessive water loss [1]. Burn injuries, chronic wounds, skin excision and other severe dermatological conditions 2 mainly cause skin damage. Any skin wound makes a passageway for microorganisms to penetrate our bodies, proliferate and cause harmful infections. Thus, dressing is necessary to protect the wound from infection and enhance wound healing.

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For the treatment of wounds, polymer-based dressing is a good choice since they allow for various forms, including hydrogel, film, membrane, nanofibers, and hydrocolloid [3]. Polyvinyl alcohol (PVA) is one of the best-known environmentally friendly polymers. It is obtained in a hydrolysis reaction of polyvinyl acetate. Its significant features are non-toxic, biocompatible, biodegradable, water-soluble, and has good film-forming ability [4, 5].

Currently, the use of medicinal plants in pharmacotherapy is receiving a lot of attention [6, 7]. Many countries have employed medicinal plant extracts because they are widely known for accelerating the wound-healing process [8]. The scientific literature is replete with reports of

medicinal plants and their benefits for improving various phases of wound healing, such as coagulation, inflammation, fibroplasias, epithelialisation, collagenation and wound contraction [9, 10]. Recently, the use of plant extracts and other alternative forms of medical treatment has been revised to be combined with updated technologies [11]. Combining bioactive herbal products with polymeric product offers great potential to create optimized biomaterials for wound healing and tissue engineering applications [12].

Several studies have shown the advantages of incorporating plant extracts into polymeric product, involving *Copaifera sp* [13], *Scutellaria baicalensis* [14], *Lithospermi radix* [15], *Cinnamomum sp* [16], *Aloe vera* [17], *Rosmarinus officinalis*, *Achillea millefolium*, *Calendula officinalis* [18], *Lavandula angustifolia*, *Mentha piperita*, *Verbena officinalis*, *Salvia officinalis folium* [19], *Urtica dioica*, *Eucalyptus citriodora*, *Chamomilla recutita*, *Stryphodedron barbatimao*, *Juniperus chinensis*, *Annona muricata*, *Curcuma longa*, *Beta vulgaris*, *Camellia sinensis*, *Centella asiatica*, *Garcinia mangostana* [20].

*Tridax procumbens* (*T. procumbens*) leaves are one of those medicinal plants. It is traditionally used in wound healing by tribal people. The matured leaves are crushed to make a paste and applied to the surface of the wound [21]. It is a weed plant that belongs to the family *Asteraceae* and is commonly known as "Coat buttons" or "Mexican daisy". It habitats waste places, roadsides and hedges. The leaves of *T. procumbens* contain 26% of crude protein, 17% of crude fiber, 39% of soluble carbohydrates, 5% of calcium oxide, 5% of Luteolin, glucoluteolin and quercetin. The leaves are traditionally used to treat wounds, high blood pressure, bronchial catarrh, dysentery, malaria, diarrhea and to prevent hair fall [22]. Studies have also reported that *T. procumbens* leaves contain antioxidant, antimicrobial, anti-inflammatory, vasorelaxant, anti-leishmanial, anti-arthritic, antihypertensive, hepatoprotective, anticancer, hemostatic, antianemic and anti-diabetic activity [23, 24]. There are only a few studies in the literature using *T. procumbens* leaves aiming at its pharmacological potential. In this line, Alkankar *et al.*, (2020) evaluated the ointment formulated with *T. procumbens* ethanolic leaf extract [25]. Ganesan *et al.*, (2017) reported the electrospun nanofibrous mat obtained from *T. procumbens* methanolic extract [26]. Trupti *et al.*, 2021 investigated the sodium alginate and pectin used to make Hemostat films loaded with *T. procumbens* extract [27]. Jude *et al.*, 2012 determined the use of the aqueous extract of *T. procumbens* leaves to treat carbon tetrachloride-induced liver injury in wistar rats [28]. Therefore, through the present work, we are the first group to have developed *T. procumbens* extract-loaded PVA films via the solvent casting method and demonstrated them as effective and sustainable wound healing agents and biocompatibility. These films of herbal origin can act as cost-efficient and reliable dressings for wound care management.

## MATERIALS AND METHODS:

**Preparation of *T. procumbens* Leaf Extract:** The *T. procumbens* plant specimen was authenticated at BSI (Botanical Survey of India), Coimbatore, India (BSI/SRC/5/23/2019/248). *T. procumbens* leaves were collected from the local farms of Tirupur district (Tamil Nadu) and cleaned thoroughly with water. After washing, the leaves were allowed to shade dry for 2-3 weeks and ground and sieved to get rid of coarse particles. 10 g of powdered sample was soaked in 100 ml of distilled water overnight at 37℃. The extract was boiled for 1 hr and filtered using Whatman No.1 filter paper. Finally, the extract was stored at 4℃ for further use [29]. Fresh leaf extract was prepared using clean, fresh leaves (10 g) ground to make a paste and boiled in 100 ml of double-distilled water for 1 hr. Then the extract was filtered using Whatman No.1 filter paper and stored at 4℃ for formulation use [30].

**Film Preparation:** Films were prepared with six different formulations by solvent casting technique according to the ratios in **Table 1** (C- Control film; D-1, D-2 and D-3: Dried leaf extract incorporated film; F-1, F-2 and F-3: Fresh leaf extract incorporated film). PVA, Glycerol prepared the film-forming solutions and different concentrations of *T. procumbens* leaf extracts (50 ml, 25 ml, and 12.5 ml). The sample devoid of leaf extract served as a control film. All the film-forming mixtures were blended by stirring on a magnetic stirrer at 60℃. The prepared films solutions were cast onto the glass petridishes and dried in an oven at 70℃.

The dried films were peeled from the casting surface and stored for further characterization studies [31]. A series of preliminary experiments were conducted to determine the optimum type and concentration of solvent and plasticizer used for preparing the PVA-based films.

**TABLE 1: COMPOSITION OF *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILMS**

| Film samples | PVA (g) | Glycerol (ml) | *T. procumbens* leaf extract incorporation (ml) | Distilled water (ml) |
|---|---|---|---|---|
| C | 5 | 0.5 | - | 100 |
| D-1 | 5 | 0.5 | 50 | 50 |
| D-2 | 5 | 0.5 | 25 | 75 |
| D-3 | 5 | 0.5 | 12.5 | 87.5 |
| F-1 | 5 | 0.5 | 50 | 50 |
| F-2 | 5 | 0.5 | 25 | 75 |
| F-3 | 5 | 0.5 | 12.5 | 87.5 |

**Hemolytic Assay:** To determine the effect of synthesised film on red blood cells, the hemolytic assay was performed [32]. 5ml of fresh human blood was collected and centrifuged at 1500 rpm for 20 min at 4℃ to obtain erythrocytes (RBC). The RBC pellet was washed three times with physiological saline and phosphate buffer saline (PBS) to remove serum proteins. After washing, the final RBC was dispersed in physiological saline to obtain an RBC suspension. The 100µl of samples with 1mg/ml concentration were taken and mixed with 850µl of PBS solution and 50µl of RBC solution. For positive control and negative control, 950µl of sterilized de-ionized water and 950µl of PBS solutions were mixed with 50µl RBC solutions. All were incubated in the dark for 10 minutes and centrifuged at 6000 rpm for 10 minutes at 4℃. The absorbance values (A) of supernatants were measured at 540 nm by a spectrophotometer. The test was performed in triplicate. The hemolysis (%) was calculated using the formula,

Hemolysis (%) = A sample – A negative control / A positive control – A negative control × 100 %

**Cytotoxicity Test:** To check the cytotoxicity of the synthesized film, a colorimetric assay called MTT assay was performed [33]. L929 cells were grown in MEM (Modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum and incubated at 37°C in 5% $Co_2$ humidified atmosphere. In 96-well culture plates, the cells were seeded at a density of $0.5 \times 10^6$ per well with synthesized film solution at a concentration range of 1mg/ml and incubated for 24 hr. After incubation, washing was done with PBS. Then MTT (methyl thiazoyl tetrazolium bromide) solution was added at a concentration of 1 mg/ml followed by incubation at 37℃ for 4 h to induce the formation of purple formazan crystals. Then, about 100µl of iso-propanol (0.04N HCl in iso-propanol) was added to each well, pipetted up and down to dissolve all of the dark blue crystals, and left at room temperature for a few minutes to ensure the dissolution of all crystals. Finally, color intensity was measured by a microplate reader at 570nm, and imaging was captured under an Inverted Phase Contrast Microscope. The data is presented as percent viability against concentration.

**Wound Scratch Assay:** The proliferative ability of the fibroblasts exposed to films containing extract was assessed using the scratch wound assay, which measures the expansion of a cell population on a surface [34]. L929 mouse fibroblast cells were seeded in 24-well plates and cultured in MEM medium supplemented with fetal bovine serum overnight.

When confluency was verified, the plate was removed, and artificial wounds were created in the monolayers by making a linear scratch in the center of each well using the tip of a sterile 200µL plastic pipette tip. Cellular debris from scratch was removed by gently washing the wells with phosphate-buffered saline (PBS).

The scratches were then divided into groups and treated with films containing 12.5 ml, 25 ml, 50 ml *T. procumbens* leaf extract in 1 mg/ml concentration. MEM medium was used as a basal control. The plates were then incubated at 37℃ in a humidified incubator with 5% $CO_2$ atmosphere. The plates were evaluated at intervals of 0, 4, 18 and 24 hour of incubation to assess the closure of the scratched area. Micrographs were used to record the wound closure activity, which was captured under an Inverted Phase Contrast microscope. After the respective time intervals, the

wound closure was measured by Image J software [35].

## RESULTS AND DISCUSSION:

**Hemolytic Assay:** Hemolysis tests are used to preliminarily evaluate the biocompatibility of the film materials. **Fig. 1** and **Fig. 2** shows the synthesized materials' hemolysis result. The control film hemolysis value is 1.87±1.73% and the hemolysis value of the films containing 50 ml, 25 ml and 12.5 ml of *T. procumbens* leaf extract values were D-1 (3.52±0.15%), D-2 (1.19±1.03%), D-3 (0%), F-1 (1.13±1.96%), F-2 (1.19±1.03%) and F-3 (0.57±0.99%) respectively.

The prepared films, consisting of PVA with *T. procumbens* leaf extract showed a hemolysis value below 5.0%. The materials whose hemolysis value is less than 5% can be considered as highly blood-compatible, which is indicative of biocompatibility. Therefore the synthesized films are suitable for wound healing applications. Similar were the results with pomegranate peel extract (PPE) incorporated PVA, poly-acrylic acid and starch film material. Hemolysis value was noted at 1.9±1.61% and 3.0±0.6% to 1.25% and 2.25% of PPE extract incorporated films, respectively [36].

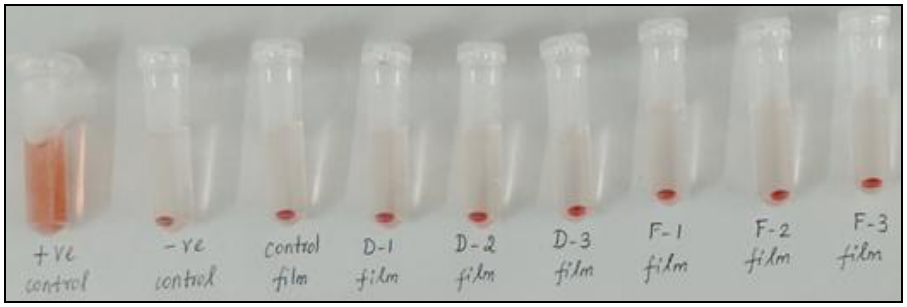


**FIG. 1: BLOOD COMPATIBILITY STUDIES OF *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM.**

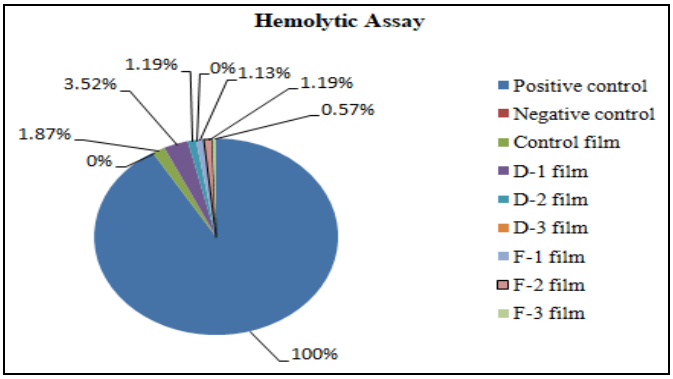


**FIG. 2: REPRESENTATION OF HEMOLYTIC VALUE OF *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM.**

**Cytotoxicity Assay:** The Cytotoxic activity of *T. procumbens* extract incorporated films was carried out by MTT assay against the L929 fibroblast cell line. Results of different concentrations of *T. procumbens* leaf extract incorporated films cytotoxicity values and microscopic images are represented in **Fig. 3** and **Fig. 4**. The highest cell viability (99%) was noted in D-3 (12.5 ml of dried leaf extract incorporated) film. The lowest cell viability (70%) was noted in D-1 (50 ml of dried leaf extract incorporated film). D-2 film showed 94% of cell viability. Fresh leaf extract incorporated film (F-1, F-2, F-3) showed cell viability above 85%. Overall, *T. procumbens* leaf extract incorporated films showed a viability range of above 70%. According to MTT assay, cell viability values above 70% can be considered non-toxic for cell lines [37, 38]. Hence, these results suggested that all the *T. procumbens* leaf extract incorporated film was non-toxic to the cells.

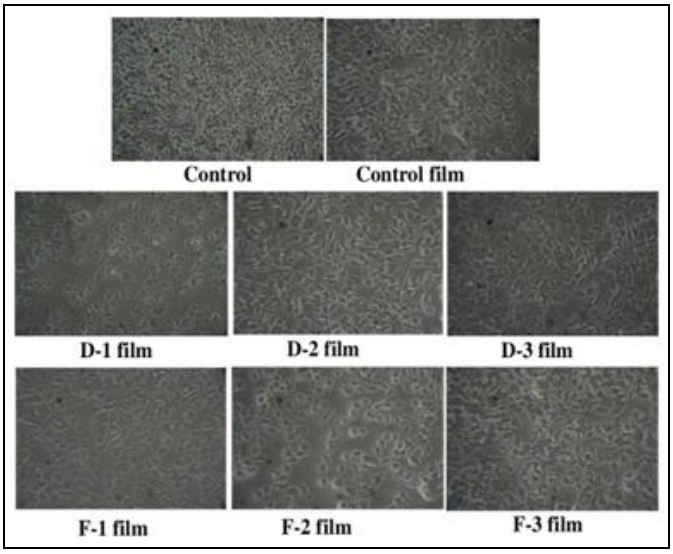


**FIG. 3: MICROSCOPIC IMAGES OF *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM IN L929 CELL LINE BY MTT ASSAY**

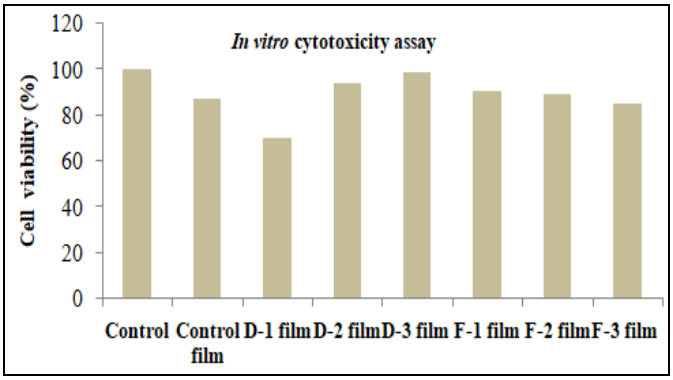


**FIG. 4: REPRESENTING PERCENTAGE OF CELLS VIABILITY AT DIFFERENT CONCENTRATIONS OF *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM IN L929 CELL LINE BY MTT ASSAY**

**Wound Scratch Assay:** In the wound healing process, cell migration provokes wound contraction, and this step is very important to trigger the later curing stages. Thus, cell migration is considered a key event in wound healing. Therefore, investigating dermal fibroblast migration after exposure to the drug formulations is an important step in developing new therapies for wound healing. The scratch assay is well-developed method to measure cell migration *in-vitro*. The wound healing efficacy of *T. procumbens* leaf extract incorporated PVA film was studied by scratch assay at 4, 18 and 24 hr time intervals. The wound healing extent of L929 mouse fibroblasts by control, control film and *T. procumbens* leaf extract loaded film (D-1, D-2, D-3 and F-1, F-2, F-3) is given in **Fig. 5**.

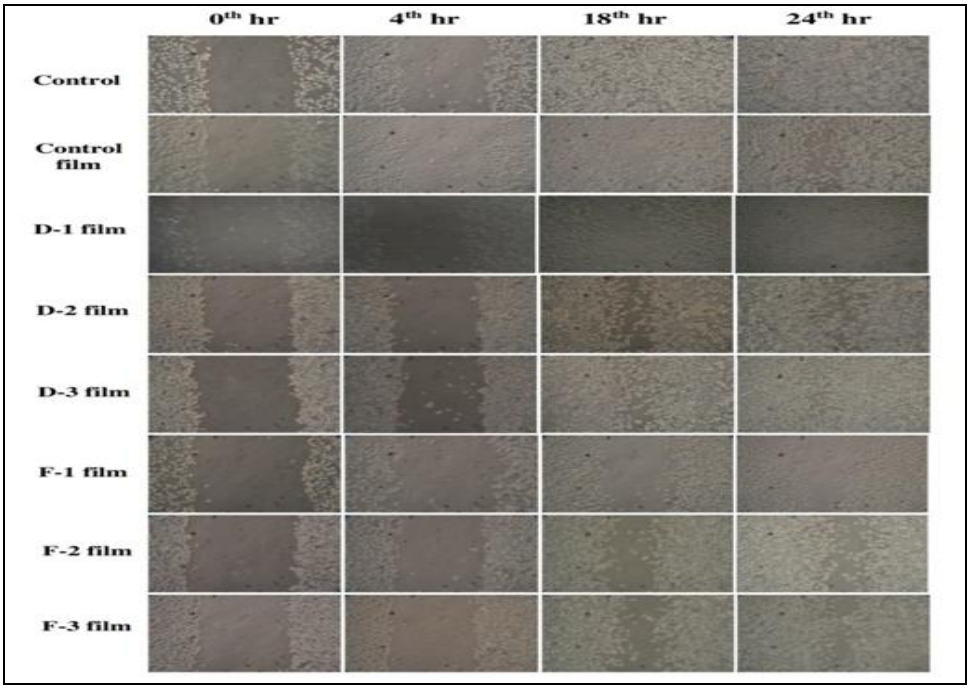


**FIG. 5: MICROSCOPIC IMAGES OF THE *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM ON CELL MIGRATION IN THE *IN-VITRO* SCRATCH ASSAY**

Likewise, the quantification of wound closure by the respective treatment was also presented in **Fig. 6**. In the control, the fibroblast cells migrated towards the scratched wound was 99.9% at 24 hrs. Whereas, the cells treated with control film accounted for 83% of wound closure at 24hrs. In case of fresh and dried leaf *T. procumbens* extract incorporated films; the wound healing effect followed the same trend. As the extract concentration increases in the film, the healing effect decreases. The lower concentration extract-loaded films (12.5 ml; D-3 & F-3) show high wound closure activity compared to the higher concentration loaded (25 ml; D-2 & F-2 and 50 ml; D-1 & F-1) films. This result trend fits the Arndt-Schulz law; a low concentration dose of an active will stimulate the cellular activity of cells, while high concentrations may inhibit cell growth [39]. Overall the dried *T. procumbens* leaf extract 12.5 ml concentrate incorporated D-3 film shows a significantly high rate of wound healing of 94% at 24 hrs. This result was similar to the study on *Moringa oleifera* leaf (MOL) aqueous extract incorporated film dressing material in which the wound healing effect was similar to the current study. The lowest concentration of 12.5µg/ml of

aqueous MOL extract attained full wound closure gap at 48 hr where it was absent, with the highest concentration of aqueous MOL extract from 25 to 100 µg/ml [39]. Interestingly, in Pinhao seed coat, nanocellulose-incorporated gels and films also showed similar results. The lowest concentration loaded films produced the efficient stimulation of cell migration compared to the highest concentration loaded films [40].

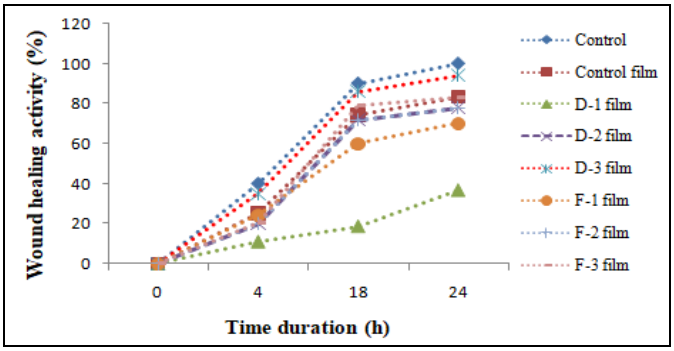


**FIG. 6: REPRESENTATION OF THE *T. PROCUMBENS* LEAF EXTRACT INCORPORATED FILM ON CELL MIGRATION IN THE *IN-VITRO* SCRATCH ASSAY**

**CONCLUSION:** Preparing innovative pharmaceutical dosage forms, such as bioactive wound dressings with natural compounds, has been gaining prominence. In this study, *T. procumbens* leaf aqueous extract was the most promising wound healing agent, as shown in the wound scratch assays. The *T. procumbens* aqueous leaf extract was successfully loaded into PVA film via solvent casting. No toxicity was observed in the *T. procumbens* leaf extract-loaded film. In the wound scratch test, compared to all other films, the 12.5 ml of *T. procumbens* dried leaf extract load film efficiently reduced the scratch open area and provoked almost complete closing of the scratches in 24 h. Future investigations on the *in-vivo* efficacy of *T. procumbens* leaf film formulations are required to establish its wound-healing potential.

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**CONFLICTS OF INTEREST:** The authors declare no conflict of interest.

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