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Macrophagic cell culture based Biomarker (Rutin) for wound healing: A New Hypothesis Approach for Cell Target

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Abstract

The present disease biomarker limits from low diagnostic sensitivity, specificity and have not yet made a major input on reducing disease burden. Because of the complex nature of biological fluids such as plasma biomarker discovery efforts using proteomics have not yet delivered any novel marker. Recently so many cell culture based model of the disease to identify novel candidate of disease biomarker. Cell secreted protein to serve as serological marker, play a central role in pathophysiology of the disease. Research has recognized numerous cellular events and mediators coupled with wound healing that can serve as biomarkers. *In-vitro* macrophagic cell culture study laid down first time with reference to assess wound healing activity. Macrophages play a key role in all stages of wound healing. In the present work an attempt was made to study effect of hydrogel containing rutin in different concentration on various haematological parameters and on the basis of above finding the macrophagic cell culture investigation was laid down. The present work showed the effect of different rutin concentrations (0.20µM, 0.25 µM & 0.30 µM) on macrophage cell culture and the result illustrated that 0.025 µM concentration drug significantly increased growth of macrophage in cell culture. Thus, it promoted wound healing activity.

Keywords: Macrophages, wound healing, rutin & biomarker.

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1. Introduction

Wounds Healing starts from the moment of injury and can prolong for varying periods of time depending on the extent of wounding and the process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue[1]. The wound healing required number of sequential steps: Initiation of an acute inflammatory process by initial injury, regeneration of parenchymal cells, migration and duplication of both parenchymal and connective tissue cells, matrix proteins synthesis, connective and parenchymal components remodeling and collagen synthesis and acquisition of wound strength [2]. Macrophages are an enormously heterogeneous group of the cells derived from circulating monocytes. Macrophages have a defensive function against pathogens and play a significant role in the homeostatic maintenance of the body through the disposal of internal waste materials and tissue repair [4]. A biomarker (biological marker) is a naturally occurring molecules used as an indicator of biological state. Proteomics is being extensively used to study molecular basis of various diseases and development of novel drugs with superior understanding of targets [5]. Several cellular events and mediators related with wound healing that can serve as biomarkers. Macrophages, fibroblasts, neutrophils and platelets release cytokines molecules including TNF-α, interleukins (ILs) and GF of which platelet-derived growth factor (PDGF) holds the significant importance [6]. After invasion of wound bed, monocytes were differentiated into macrophages. Macrophages play an essential role in all stages of wound healing. Their functional phenotype depends on the wound microenvironment, which changes during healing, thereby altering macrophage phenotype.

Macrophages exert pro-inflammatory functions like antigen-presenting, phagocytosis and the production of inflammatory cytokines and growth factors that facilitate the wound healing during short inflammatory phase. Macrophages stimulate proliferation of connective, endothelial and epithelial tissue directly and indirectly during the proliferative phase [7]. Fibroblasts, keratinocytes and endothelial cells are stimulated especially by macrophages during this phase to induce and complete ECM formation, re-epithelialization and neovascularization.

Phases of Wound Healing [3]

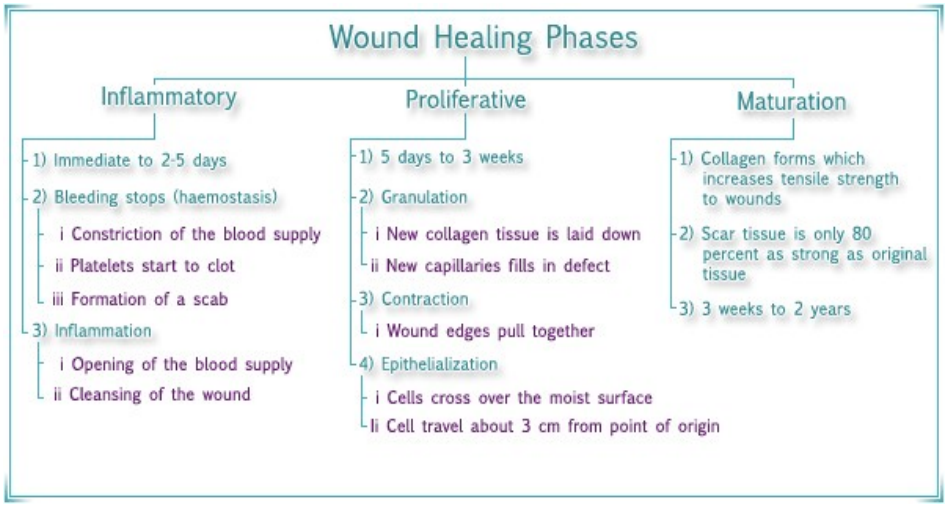
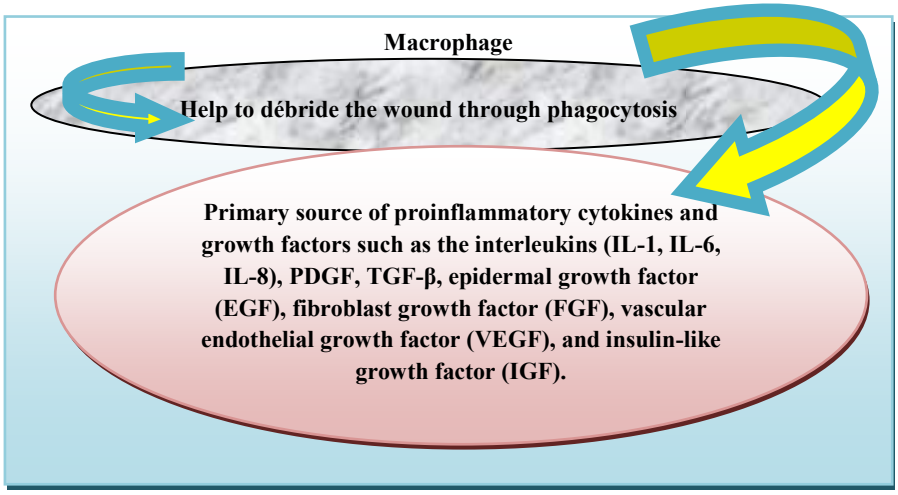


Figure 2: Role of macrrophage in wound healing



Rutin is quercetin-3-rutinoside or 3,3',4', 5,7-pentahydroxy flavones-3-rutinoside having chemical formula $C_{27}H_{30}O_{16}$ i.e. rhamnoglucoside of the flavonoid quercetin and found in many plants and used for treatment of various diseases related to the vascular [8].

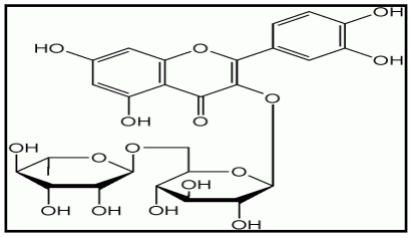


Figure 3: Structure of Rutin

In the present work an attempt was made to study effect of hydrogel containing rutin in different concentration on various haematological parameters and on the basis of above finding the macrophagic cell culture investigation was laid down. Thereby an attempt has been made to identify the biomarker with reference to wound healing activity with the help of macrophagic cell culture.

2. Material and methods

2.1 Material

Rutin was previous isolated from leaves of *A. squamosa* and characterized by chromatographic as well as various modern analytical methods like FT-IR, NMR & Mass spectroscopy.

RPMI media was purchased from Hi- media Lab, Bilaspur (C.G.).

2.2 Experimental Protocol

2.2.1 Selection and procurement of animals

Albino rats were procured and rats of either sex (weighing 150-200 g) were selected, maintained at 24-28^{0}C, housed individually with free access to food and water. Rats were fed standard diet and kept in well-ventilated animal house with alternate dark-light cycle of 12 hrs throughout the studies (CPCSEA-1323/10-CPCSEA).

2.2.2 Hematological analysis

To perform the experiment, the mice were divided into five groups consisting of six animals each.

Group I - CONTROL
Group II - TEST (H1)
Group V - TEST (H2)
Group VI - TEST (H3)
Group V - Standard [Standard]

All the samples were applied once daily for 16 days, starting from the day of wounding. After 16 days blood sample was collected from retro-orbital cavity and evaluated for the hematological parameter.

***2.2.3 In- vitro* macrophage cell culture study**

The culture medium and glasswares were kept in a refrigerator to prevent sticking to the glass surface.One rat(1-2 month old) was taken and scrificed by cervical dislocation. The rat was pinned on board with abdomen position kept upward then was hed it with 70 % v/v ethanol.Skin was lifted from the abdominal wall with the help of forcep,a median longitudinal cut was made and separated the outer skin to expose the abdominal wall. 2.5 ml of culture medium was injected along the mid anterior line avoiding puncturing the gut, after which the abdominal wall is ballooned up. With the help of fingertips gently massaged the abdominal wall to release the macrophage from the cell membrane. Now the U-100 insulin needle was inserted in flank and the fluid was slowly aspirated.The fluid was transfed in chilleed sterile ependroff tubes & centrifuged at 100 RPM for 10 min and resuspended the cell in 10 ml RPMI -1640 growth medium. These suspended cells in growth medium were counted, and adjusted the cell density at 1X10^4 cells/ml. After optimum O.D. cell culture distributed in cell culture vessele and incubated in humidifier contain 5% CO_2 at 37^{0}C. Cell suspension was filled in 96 wells microtitre plate according to table plan, then incubated in humidifier contain 5% CO_2 at 37^{0}C for 48 hr. Finally observe the OD at 560nm by BioRed ELISA Reader and interpretate the result to check the effect of drug on cell culture.

2.3 Statistical Analysis

The data was statistically analyzed by one-way analysis of variance (ANOVA) using Graph Pad software. The difference was considered significant when *P*-values < 0.05. All the values were expressed as mean±standard error mean (S.E.M.).

Table 1: Effect of Hydrogel containing rutin in different concentration in Hematological parameter

Parameter	Control	H1(0.020%w/w)	H2(0.025%w/w)	H3(0.030%w/w)
WBC(x103 cells/µL)	5.91±0.17	7.67±1.89	8.02±2.21	8.3±1.32
RBC(x106 cell/µL)	6.11±0.27	6.98±0.43	7.56±0.21	7.62±0.14
Platelet(x103 cells/µL)	297±98.12	323±109.23	396.21±104.29	393.15±107.24
Monocytes(%)	5.72±2.8	5.29±1.78	6.30±2.3	6.5±2.7
GR (%)	8.97±3.49	9.83±2.42	10.21±3.38	10.4±3.16

Figure 4: Blood Cells Analysis of Rutin containing Hydrogel

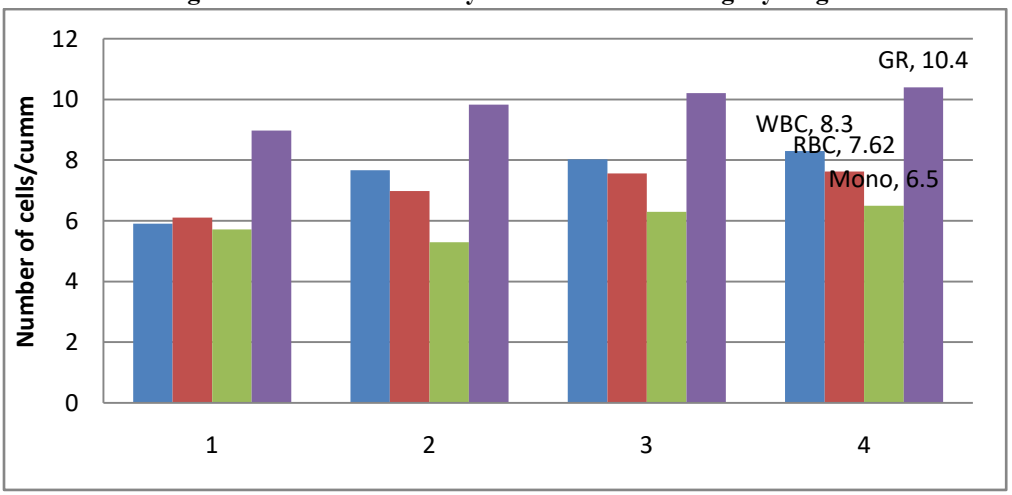


Figure 5: Platelets analysis of Rutin containing Hydrogel

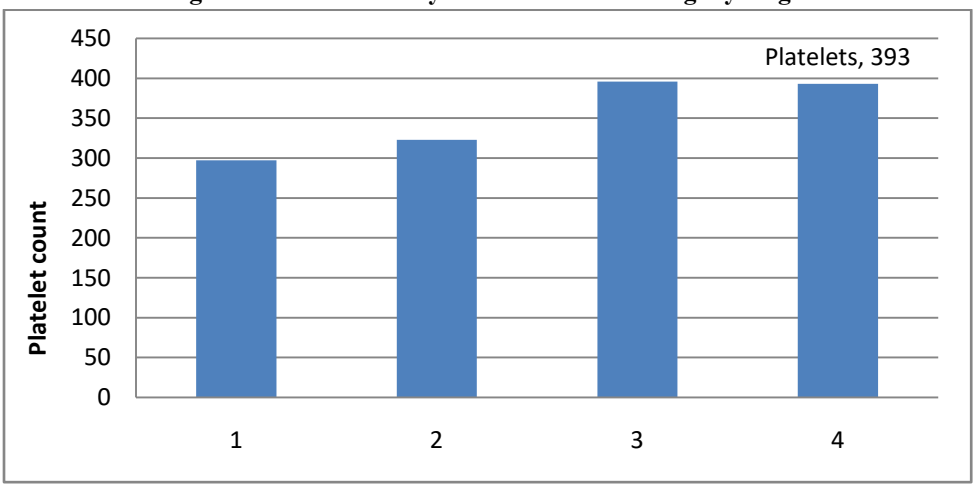
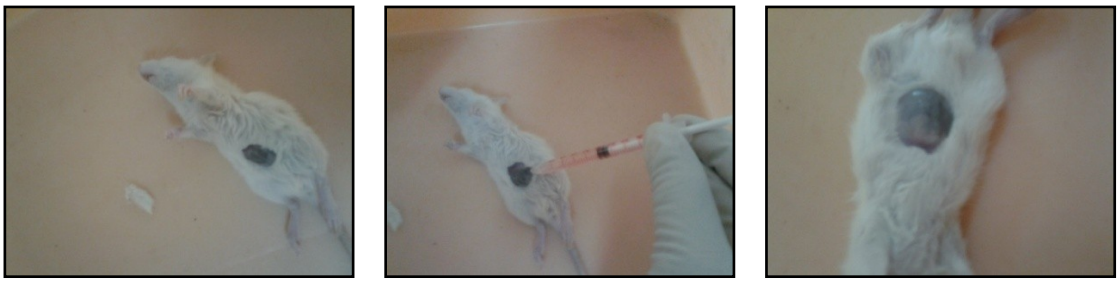


Figure 6: Hematological analysis of rutin containing hydrogel



a) **Cutted abdominal wall** **b) Injecting media by U-100 needle** **c) Balooned abdominal wall**

a) Aspirating Macrophagic fluid **b)aspirated macrophage in media**

Figure 7: Isolation of macrophage from rat

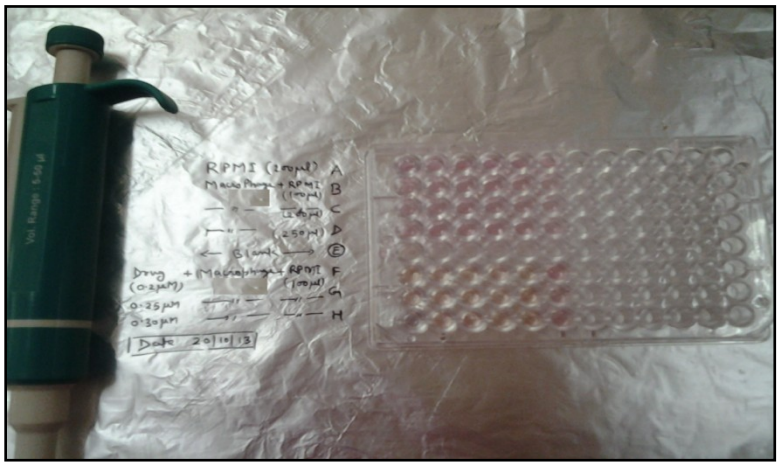


Figure 8: Preparation of 96-well plate for macrophage cell culture

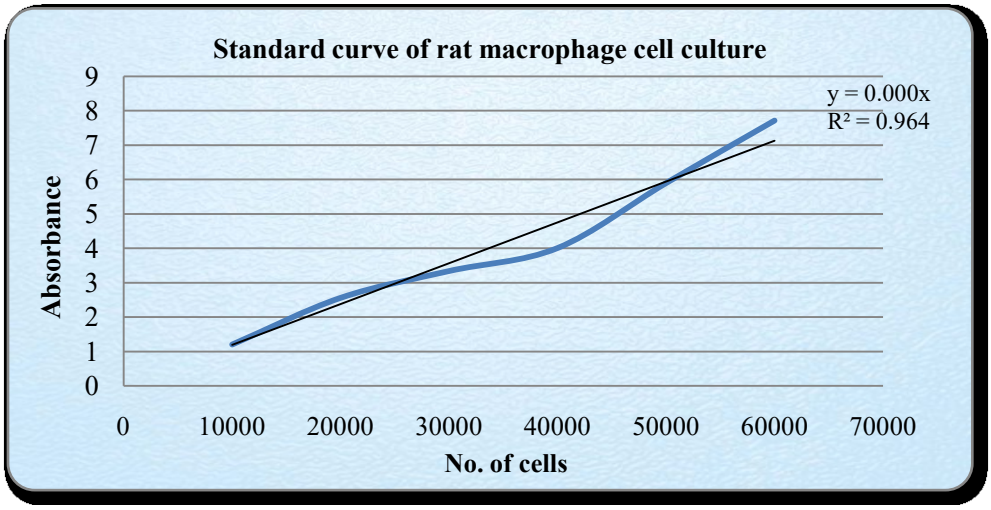


Figure 9: Standard curve of rat macrophage cell culture

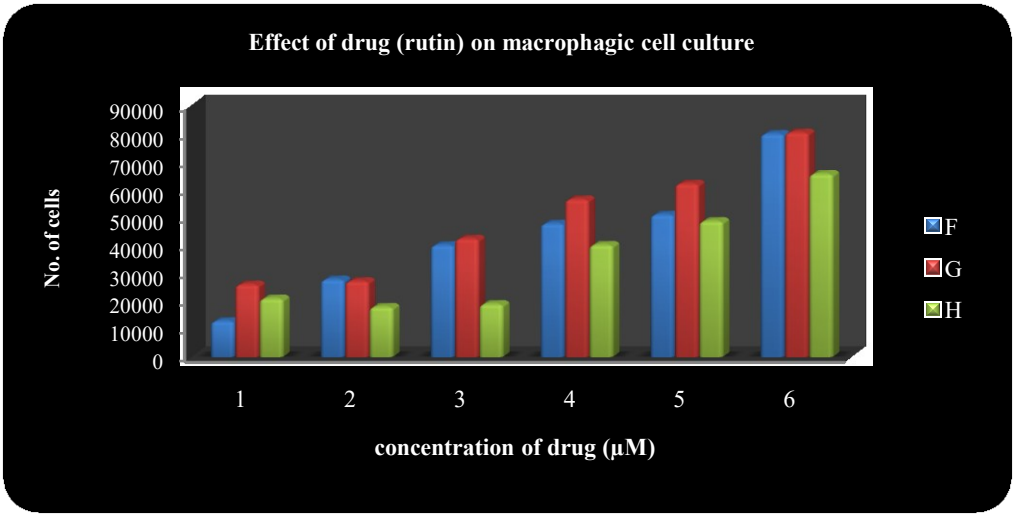
Table 2: Observation of rats Macrophage cell culture (Micro titre plate reading Report at 560nm)

	Groups	Wells					
A	Control(RPMI)	2.8	2.812	2.804	2.811	2.81	2.9
B*	M cell(10-60µL) + RPMI(100µL)	**1.2**	**2.56**	**3.34**	**4.01**	**5.90**	**7.72**
C	M cell (10-60µL)+ RPMI(200µL)	1.31	2.72	3.90	4.04	5.94	7.81
D	M cell (10-60µL)+ RPMI(250µL)	1.01	1.92	2.03	2.84	4.43	6.70
E	Blank	00	00	00	00	00	00
F	M cell (10-60µL)+ RPMI + 0.20µM (Rutin)	1.29	2.78	4.01	4.78	5.10	7.99
G*	M cell(10-60µL) + RPMI + 0.25µM (Rutin)	**2.6**	**2.72**	**4.24**	**5.65**	**6.21**	**8.04**
H	M cell(10-60µL) + RPMI + 0.30µM (Rutin)	2.1	1.8	1.9	4.02	4.87	6.56

M cell = microphage cell

Table 3: Effect of Rutin on macrophagic cell culture with reference to cell number

Drug treated culture	No. of Cells					
F	1.29×10^4	2.78×10^4	4.01×10^4	4.78×10^4	5.10×10^4	7.99×10^4
G*	2.6×10^4	$2.72X10^4$	$4.24 X10^4$	$5.65 X10^4$	$6.21 X10^4$	$8.04 X10^4$
H	2.1×10^4	1.8×10^4	1.9×10^4	4.02×10^4	4.87×10^4	6.56×10^4



Fiure 10: Effect of drug (rutin) on macrophagic cell culture

3. Result and Discussion

Rutin (flavonoid) is a potent antioxidant. Rutin was reported to hinder neutrophil infiltration and to modulate nitric oxide production. Nitric oxide is formed from the L-arginine (amino acid) and oxygen by three distinct isoforms of nitric oxide synthase [9]. The inducible isoform (i-NOS) is synthesized in the early phase of wound healing by inflammatory cells (mainly macrophages) and is a critical co-factor in wound healing. NO serves as an important biomarker for a diagnostic test for wound

healing. Platelets make available numerous of bioactive proteins which plays key roles during healing process include homeostasis and inducing enrollment of WBCs[10]. Inflammation is one of the stage of the wound healing process [11]. In the initial phase of tissue repair, platelets appear at a wound site, degranulate, and release their various contents, inclusive of GF. They also provide proteins for hemostasis including fibrinogen. The vital role of WBCs in wound healing and tissue repair has been recognized for decades. The hematological parameters studied after 16th of application of the topical hydrogel formulation containing different concentration of rutin showed increase in WBC and RBC count. The result revealed that H3 and H2 formulation showed more or less similar but concentration of drug in H2 is less than H3, which conclude that H2 formulation give significant out come on hematological profile (Table .1 and Graph.1). On the basis of above finding and with the assist of literature survey an attempt was made to study effect of rutin in the macrophagic culture. *In-vitro* macrophagic cell culture study laid down first time with reference to assess wound healing activity. Macrophages play a key role in all stages of wound healing. The effect of different rutin concentrations (0.20µM, 0.25 µM & 0.30 µM) on macrophage cell culture illustrated that 0.025 µM concentration drug significantly increased growth of macrophage in cell culture (Table 3 & 3 and graph 2 & 3). Thus, it promoted wound healing activity.

4. Conclusion

From the macrophagic cell culture study, it can be concluded that rutin possess plethora of a range of biological effects. The modulating effects of rutin on various stage of wound repair showed that they reduce the time required for wound closure. It also reduces the oxidative stress in wound area as signified by a reduction in lipid peroxidation. In the present investigation rutin significantly increases macrophage in cell culture thereby it enhance production and accumulation of ECM along with cell proliferation. Author already proved that the indication of two molecular combinations and their efficacy against the pro-inflammatory protein scaffolds. Increased paw diameter and lysosomal enzyme activity in the arthritic animals were significantly suppressed to near normal levels in rats [12]. Hypothetically it is possible that macrophages are necessary for the production of a various factors that promote fibroblast proliferation. The biology of blood cells is complex, as they are involved in both inflammation and tissue healing. Rutin has showed reduction of inflammation in rat model and its hydrogel formulation give the sufficient opportunity to justify the preclinical indication to treat the target diseases. Since chronic wounds are often the result of excessive inflammation, controlling the immune response is an attractive avenue for designing novel regenerative strategies. To this end, numerous material-based and molecular strategies have been explored; including targeting the immune response using cytokines, protease inhibitors, miRNA, small interfering RNA (siRNA) and extracellular vesicles (EVs). Over the past decade, considerable insights into the molecular pathways driving the animal healing response and impairment have suggested new therapeutic targets and provided scientific rationale for future clinical trials.

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