Original Article

Simultaneous Quantification of Quercetin and Syringic Acid in Methanolic Extract of *Leucas lavandulifolia* by using Validated HPTLC-Densitometric Method

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ABSTRACT

A simple, sensitive and reliable High Performance Thin Layer Chromatography (HPTLC) method was developed for the simultaneous quantification of bioactive compounds *i.e* quercetin and syringic acid in *Leucas lavandulifolia.* Methanolic extract used on HPTLC silica gel 60 F254, 10 × 10 (Mark) plate for the separation and quantification of bioactive compounds by using toluene: ethyl acetate: formic acid (7: 2.5: 0.5 v/v) as mobile phase. The used mobile system gives well-resolved bands of quercetin (Rf value 0.32 ± 0.06) and syringic acid (R_f value 0.38 ± 0.05) in the extract of *L. lavandulifolia*. The correlation coefficient was found to be 0.987 and 0.997 with standard deviation of 667.77 and 3819.77 for quercetin and syringic acid. The accuracy of the method was confirmed by recovery studies at different levels using the standard addition method. Precision studies (both inter day and intraday) are within the standard limit, RSD (%) is less than 3%. The average recovery of quercetin and syringic acid was 100.02% and 100.13% suggesting the accurateness of the method. The quantification of quercetin and syringic carried out by using a densitometric absorption mode at 275 nm and 370 nm respectively. The developed method was validated and found to be specific, linear and accurate with precision and accuracy in the concentration range of 2.0–6.0 µg/spot for quercetin and syringic acid. Hence, simple, reproducible and selective HPTLC method has been developed for the estimation of the identified bioactive markers which may be utilized as a tool for the proper authentication and standardization of *L. lavandulifolia.*

Key words: HPTLC, *Leucas lavandulifolia*, Quercetin, Syringic acid, DPPH, Hydroxyl Radical-Scavenging.

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INTRODUCTION

Nature has provided us a huge amount of remedies to heal illness of human being. In today's scenario, there is an increasing interest in herbal medicines accompanied by increased research investigation into the pharmacological properties of phytochemical ingredients and their capacity to treat various types of diseases.[1,2] A number of drugs have been entered in the international market which had been previously used in ethanomedicine and traditional medicine. Although, scientific study have been carried out on a large scale to provide the evidence-based therapeutics data for the authentication and validation of ayurvedic drugs. In the Indian context, 43 species of genus *Leucas* are available, generally shrubs, sub-shrubs, annual herbs, or perennial herbs with woody root and/or stem base.[3] The genus *Leucas lavandulifolia* Sn. (Lamiaceae) has been widely in use by the traditional healers to cure many diseased which shows that this genus has huge potential for the discovery of new drugs. The *Leucas lavandulifolia* (*Leucas indica*) commonly known as Gumo (Hindi), Halkusa (Bengali) is a very well known medicinal plant in Indian system of

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medicine from time immemorial.[4] It is a herbaceous, annual weed, 30-80 cm tall, found in grazing land, wasteland and in roadside areas[5] (Anonyomous, 1962) and it is widely distributed throughout Bangladesh, Sri Lanka, Nepal, Myanmar, Thailand, Malaysia, Indonesia to new Guinea, China, tropical American countries and India. In India it is found in Bihar, UP Andhra Pradesh, Kerala and Tamil Nadu.[6]

Various phytochemicals in *Leucas lavandulifolia* were also reported such as linifolioside, isopimarane rhamnoglucoside, leupol, taxaxerone and chrysoeriol-6. Evaluation of tincture in *L. lavandulifolia* was characterized by examination of co-chemical properties of *L. lavandulifolia* (Arial part). Some phytochemical such as acacetin, chrysoeriol were also investigated in chloroform and diethyl extract of the species.[7, 8]

Traditionally, it has been extensively used by rural people of Bihar especially in Mithila region, past of tender shoots is applied to relieve headache, leaf juice used to treat stomach complaints, poisonous insect bites, skin diseases, scorpion sting, cough and cold.[9] The local tribes of Orissa used this plant as a vegetable. The juice of the plant is used in the treatment of malarial fever.[10] Whole plant powders of *Leucas lavandulifolia* and *Wattakaka volubilis* are mixed with water and orally administered thrice a day for curing paralysis and fits. Pharmacologically, *L. lavandulifolia* is used in antitussive, wound healing, hypoglycaemic,[11, 12] anti-inflammatory and antibacterial,[13] as reported in the literature. The phytochemical analysis shows the presence of carbohydrate, alkaloids, steroids, flavonoids, triterpenoids, essential oil, saponins and tannins.[14-16] Apart from this isopimarane rhamnoglucoside, linifolioside, linifoliol,[17] lupeol, taraxerone have been isolated from the aerial parts of the species.[18]

In view of its diverse therapeutic efficacy and traditional use in India, it was desirable to fix some pharmacognostical parameters for future identification of the drug materials to explore its different phytochemical constituents. In the present study, we are focusing on identification and quantification of quercetin and syringic acid in *Leucas lavandulifolia* and evaluation of its antioxidant potential. This study will be helpful in the authentication and identification of the particular plant used as a drug in pharmaceutical industries.

MATERIALS AND METHODS

Plant material

Collection of *Leucas lavandulifolia* was done in September 2014 from Tamiya, Madhya Pradesh, India. Plant material was identified and authenticated by Dr. Sharad Srivastava, Senior Principal Scientist Pharmacognosy division, CSIR-NBRI, Lucknow. Plant specimen was assigned a voucher number (LWG No. 254034) and deposited in the institute's repository. Arial parts were washed, shade dried and powdered (40 mesh) using an electric grinder (Figure 1).

Chemicals

Toluene, ethyl acetate, methanol, formic acid (MP biomedical Ltd.) and HPTLC precoated silica gel 60 GF_{254} (10 x 10 cm) plates (Merck, India) was used. Ascorbic acid, quercetin, rutin, BHT (Butylated hydroxy toluene) and 1-1-diphenyl-2-pic-rylhydrazyl (DPPH), 2-deoxy ribose sugar, Quercetin (95%), syringic acid (97%) were purchased from Sigma-Aldrich. All other chemical were purched from SD fine chemicals.

Extraction protocol for Quercetin and Syringic acid

The coarsely powdered aerial parts of *Leucas lavandulifolia* (2 gm) were macerated with methanol for 24 hrs at room temperature (25 ± 2°C). Extraction repeated thrice, filter and pooled filtrate was dried in rotatory evaporator (Buchi, USA) under standard conditions of temperature (55 ± 2°C), pressure (40 mbar) and then lyophilized (Labconco, USA). The extractive yields were calculated 368 mg on a dry weight basis.

Chromatographic Conditions

A stock solution of sample and the markers of strength 10 mg/mL and 1 mg/mL respectively were prepared in methanol. Working solution of sample (1.0 mg/mL) and standards (quercetin and syringic acid) (0.1 mg/mL) were freshly prepared from stock solution for analytical work. The spot was applied as 8 mm wide bands positioned 12 mm from the bottom and 18 mm from the side of the plate, using CAMAG Automated TLC Sampler 4 (ATS 4) with nitrogen flow providing a delivery speed of 150nl/s from application syringe. These conditions were kept constant throughout the analysis of samples. The plate was developed in a CAMAG Automatic Developing Chamber (ADC) by using standard developing condition, 30 min saturation time of the chamber, controlled humidity ($MgCl_{3,}$ 33%RH), 25°C room temperature, 85 mm solvent run from bottom to top of the plate, by putting 10 mL tertiary mobile phase (solvent system) in the ratio of toluene: ethyl acetate: formic acid (7: 2.5: 0.5 v/v) on twin trough developing chamber (20 x 10 cm). 10 min TLC plate was dried and scanned by using densitometory CAMAG TLC scanner equipped with Vision Cats software (slit width, 5 mm x 0.30 mm) in absorption reflection mode.

The quantification of quercetin and syringic acid were carried out using a densitometric absorption mode at 275nm and 370 nm (Figure 2 and 3).

Method validation

HPTLC method validation includes evaluation of linearity, sensitivity, precision, selectivity and robustness parameters according to the ICH guidelines, 2005, to access the performance of the method.[19]

Linearity

Different dilutions were spotted in triplicate on TLC plate concentrations of 2, 4, 6, µg per spot of quercetin and syringic acid (Table 1). The data of peak area versus concentration were treated by the linear least-square regression equation. The slope, intercept and correlation coefficient for the calibration curve were determined with 3 different concentrations. The results are expressed as a percentage of the total area of identified compounds. Based on the calibration curve quercetin and syringic acid content was estimated in the plant and expressed on a dry weight basis.

Sensitivity

The sensitivity of the method was determined with respect to Limit of Detection (LOD) and Limit of Quantification (LOQ). It was calculated are as signal noise ratios 3:1 and 10:1 (Table 1).

Stability

The reproducibility of the method was determined by analyzing standard of single concentration (0.1 mg/mL) over three times on the same day. The relative standard deviation was used to evaluate the reproducibility of the method within the limit of the standard. The developed method was also validated for selectivity, specificity and resolution of the analytic (Table 1).

Precision

Interday and intraday studies were carried out to test the precision of method and expressed as the relative standard deviation (%). Intraday repeatability was tested by injecting sample solution of individual population three times a day. Similarly, interday repeatability was assessed over three consecutive days by using same concentration (of the sample), thrice a day (Table 3).

Accuracy

The accuracy (standard addition method) of the methods was determined by analyzing the percentage recoveries and mean RSD (%) of standard quercetin and syringic acid in the collected samples (Table 4). The samples were spiked with three different concentrations: 50, 100 and 150 µg. The spiked samples were recovered in triplicate and then analyzed by developed the HPTLC method.

Robustness

Robustness is evaluation of the method to stay unaltered by small but deliberate variations in the experiential conditions, which indicate of the reliability of the method. It was achieved by introducing small variations in chromatographic parameters, such as small variations in the ratio of the mobile phase, the time gaps between spotting to chromatography and from chromatography to scanning and the time interval between drying and scanning

In-vitro Antioxidant Activity

Antioxidant potential of *Leucas lavandulifolia* was determined by DPPH radical scavenging assay[2-] and Hydroxyl Radical-Scavenging Activity.[21]

Statistical analyses

Observations of each sample were performed in triplicate. The data were recorded as mean ± standard

Table 1: Calibration parameter of Quercetin and Syringic acid.

Sl.No.	Parameter	Quercetin	Syringic acid
1	Linearity range (ng/spot)	2000-6000	2000-6000
2	Rf (cm)	0.32	0.41
3	λ_{max}	275nm	370nm
4	Regression Equation	y = 329650x + 28720	y = 329650x + 28720
5	Correlation coefficient	0.987	0.997
6	Regression coefficient	0.9748	0.9952
7	Average	30038.57	17730.8
8	Slope	329.65	1905.32
9	Standard deviation	667.77	3819.776
10	Standard error	149.99	373.1798
11	LOD (µg/spot)	20.25	102.36
12	LOQ (µg/spot)	6.68	33.77
13	Intercept	28719.97	10109.5

Table 2: Quantification of quercetin and syringic acid in *Leucas lavandulifolia*.

S/N	Analysis	Value (% dry weight)
1	Quercetin	0.15 ± 0.002
2	Syringic acid	0.45 ± 0.005

n=3, ± SD (Standard deviation)

deviations and analysis of variance (ANOVA) was used to calculate the critical f value (*t*-test) and the statistical significance for the analyzed of quercetin and syringic acid content by Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA). The significance of the regression coefficients was evaluated by f test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Calibration

Calibration curve was achieved by using linearity range 2000-6000 ng/spot with equation y = 329.65x + 28720, regression coefficient (R^2) = 0.9748 and y = 1905.3x + 10110, regression coefficient (R^2) = 0.9952 of quercetin and syringic acid respectively. Slope and standard deviation of quercetin and syringic acid was found 329.65, 1905.32 and 667.77, 3819.776 respectively (Table 1). LOD (3:1) and LOQ (10:1) values were within the limit of acceptance. Other statistical parameters were in accordance with ICH guidelines.

Quantification of marker compounds

As per the previous studies, bioactive compounds are mainly responsible for the biological potential of any species.[22-24] Thus, to validate the claim it is necessary to identify and quantify the bioactive markers which confirm the potential of the species. For this, simple, accurate and stable HPTLC chromatography method was developed for the quantification of quercetin and syringic. The mobile phase used for the separation of quercetin and syringic acid is toluene: ethyl acetate: formic acid (7: 2.5: 0.5 v/v), shows the proper separation and better visibility in the UV-light compare to the other mobile phases which has been reported in earlier studies. Bands for the quercetin and syringic acid present in the samples were compared with the R_f value and the UV spectra of the marker compounds (Figure 2 and 3). The R_f value of these markers was found (0.32±0.002) for quercetin and in (0.31±0.003) for syringic acid. The dry weight percentage of the marker compounds viz - quercetin (0.15±0.002) % dry weight and syringic acid (0.45±0.005) % dry weight present in the aerial parts of *Leucas lavandulifolia* respectively (Table 2).

Method validation

The method was calibrated at three different dilutions of quercetin and syringic acid (200, 400 and 600 ng/spot) with linear regression equation {(y = 329650x + 28720) and (y = 329650x + 28720) respectively} and regression coefficient t (0.9748 and 0.9952 respectively)

Table 3: Intraday and Interday study of quercetin and syringic acid.

Concentration (ng spot^{-1}) Amount of Standard	Quercetin				Syringic acid			
	Intra Day		Inter Day		Intra Day		Inter Day	
	SD	%RSD	SD	%RSD	SD	%RSD	SD	%RSD
2000	5.139	0.017	06.472	0.0237	18.449	0.1342	11.296	0.081
4000	7.807	0.026	13.016	0.0248	12.232	0.067	20.177	0.104
6000	18.79	0.061	09.013	0.0196	35.652	0.167	13.697	0.0632

n=3, ± SD (Standard deviation)

Table: 4 Result and statistical data for recovery studies for quercetin and syringic acid in *Leucas lavandulifolia*.

Sample	Amount of quercetin present in Sample (µg)	Amount of quercetin Added (µg)	Theoretical added value (µg)	Amount of quercetin analyzed (µg)	Recovery (%)	Average Recovery	Amount of syringic acid present in Sample (µg)	Amount of syringic acid Added (µg)	Theoretical added value (µg)	Amount of syringic acid analyzed (µg)	Recovery (%)	Average Recovery
Leucas lavandulifolia	0.088	50	50.088	50.095	100.01	100.016	0.473	50	50.473	50.481	100.01	100.13
	0.088	100	100.088	100.12	100.03		0.473	100	100.473	100.631	100.15	
	0.088	150	150.088	150.11	100.01		0.473	150	150.473	150.824	100.23	

n=3, ± SD (Standard deviation)

were obtained respectively (Table 1). Calibration curve for quercetin and syringic acid (area vs. concentration) shows the positive random pattern, indicating that a linear model provides a decent fit to the data.

According to the ICH guidelines, 2005 various parameters *viz.* the limit of detection (LOD), the limit of quantification (LOQ), linearity, sensitivity, precision, selectivity and robustness were studies to validate the method. LOD (3:1) and LOQ (10:1) values are within the limit of acceptance. Other statistical parameters are in accordance with ICH guidelines as shown in (Table 3 and Table 4). Stability of method was evaluated by repeated (n = 3) analysis of standard at single level (0.1 mg/mL), standard deviation (667.77, 3819.77), standard error (329.65, 1905.32) and intercept (28719.97, 10109.5) reveals that method is stable under chromatographic conditions. For specificity, UV spectrum of samples was analyzed and, was found to be superimposed over the reference standard and peak was obtain at same R_f (Figure 3). The peak purity of these compounds was also assessed by comparing the spectra at three points' viz., peak start, peak apex and peak end positions (Figure 4) Precision validation of method was analyzed by interday and intraday repeatability studies at single level using fixed concentration (0.1 mg/mL) of standard solution. RSD (%) values as shown in are observed within the limit i.e. NMT 5 %. Accuracy is tested through standard addition method by spiking of samples at three different levels of 50, 100 and 150 % (Table 3). Recovery of analyte shows the variation from 100.03 to 100.01% (quercetin) and 100.23 to 100.01% (syringic acid), which are in the acceptance limit of 95-105 % and hence the method was found to be accurate and precise also (Table 4). Method robustness is tested by deliberate variations in the method conditions and indicates the reliability of the method for the robustness study, different mobile phase compositions and injection times were assessed. Developed HPTLC method is simple, reliable, accurate and reproducible for the quantification of targeted marker compounds.

In-vitro assay

In-vitro antioxidant potential

In previous study, phenolic and flavonoid compounds demonstrated high antioxidant potential in various *in vitro* and *in vivo* systems.[25] By using these compounds, plants developed effective defense mechanism of the system against harmful effects, to protect from visible

Figure 1: A flowering twig of *Leucas lavandulifolia*.

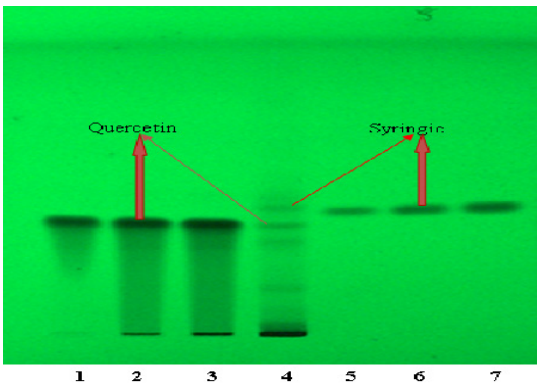

Figure 2: HPTLC fingerprinting of *Leucas lavandulifolia* at 254nm.

Abbreviation: 1-3 Quercetin, 4- Leucas lavandulifolia, 5-7 Syringic acid

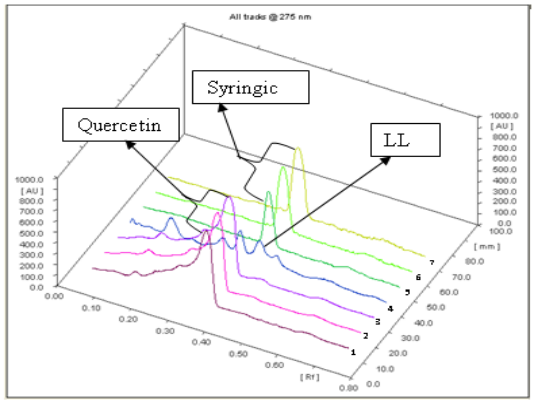

Figure 3: 3D Densitometry analysis of *Leucas lavandulifolia*.

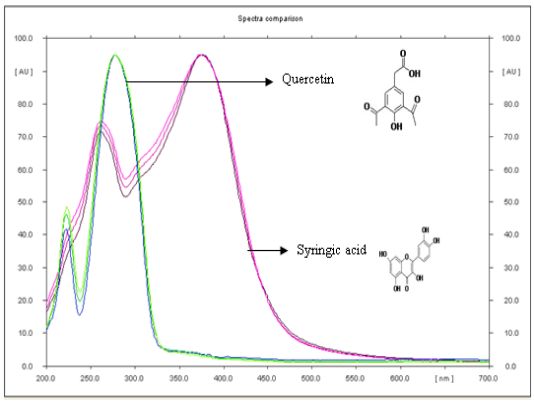

Figure 4: Purity Spectral analyses of quercetin and syringic acid.

Table 5: Phytochemical analysis of *Leucas lavandulifolia.*

S/N	Analysis	Value (mg/g)
1	Total Phenolic content {(mg/g)*GAE}	5.6±0.004
2	Total Flavonoid content {(mg/g)*QE}	4.22±0.007

n=3, ± SD (Standard deviation)

and invisible aspects.[26,27] Quercetin and Syringic acid are phenolic compounds which prevent the endogenous formation of free radicals which ensured by the spatial separation of the process in which free radicals are formed.[28] It also inhibits xanthine oxidases (XOD) which have the ability to capture superoxide radicals. It also helps in the treatment of other diseases such as gout and ischemia by reducing uric acid and superoxide radical.[29]

Total phenolic and flavonoid analysis was performed in the aerial part of *Leucas lavandulifolia* which shows phenolic content (TPC) 5.6±0.004 {(mg/g)*GAE} and total flavonoid content (TFC) 4.22±0.007 {(mg/g) *QE} (Table 5). Previous antioxidant study of *Leucas* species shows that ethanolic extract has huge potential against DPPH and free radical scanning assay and data directly correlated with phenolic and flavonoid content.[30-31] In our previous study, four *Leucas* species (*L. aspera*, *L. biflora*, *L. cristata* and *L. mollissima*) aerial part were analyzed and found that *L. aspera* have highest phenolic and flavonoid content among of them.[32] In present investigation, i*n vitro* antioxidant activity was evaluated by DPPH and deoxyribose method. Data reveals that activity increases linearly with concentration, i.e., 0.1–0.5 mg/mL of tested plant extract. IC_{50} value found as followed by (404.1 ± 0.003) and (9.601 ± 0.033) in DPPH and deoxyribose assay respectively (Table 6). Pharmacological potential of the plant mainly depend on the quantity of the bioactive compounds which ultimately directly affect the potential of the species. In our previous investigation, four *Leucas* species were selected to find out the best antioxidant potent of the species. In this, *Leucas aspera* shows high phenolic and flavonoid content along with antioxidant potential. In present investigation, *Leucas lavandulifolia* shows more phenolic, flavonoid content and antioxidant potential, compared to the *L. aspera*. Within the confinement of our study, we conclude that *L. lavandulifolia* showed excellent antioxidant potential with reference to other species *L. aspera*, *L. biflora*, *L. cristata* and *L. mollissima*. Therefore, this study may be helpful in future for the selection of potential *Leucas* species and also may be used as alternative species in preparation of ayurvedic formulations.

Table 6: Analysis of antioxidant potential of *Leucas lavandulifolia.*

S/N	Plant/standard	DPPH {IC_{50} (µg/mL)}	Deoxy ribose assay {IC_{50} (µg/mL)}
1	*Leucas lavandulifolia*	404.1 ± 0.003	9.601 ± 0.033
2	Quercetin	06.04 ± 0.122	07.55 ± 0.021
3	Syringic acid	05.241 ± 0.173	06.52 ± 0.074
4	Ascorbic acid	3.86 ± 0.057	10.37 ± 0.057

n=3, ± SD (Standard deviation)

CONCLUSION

Essential metabolites are present in considerable amount viz- flavonoids, phenolics which supports the *in-vitro* biological screening and establishes standards of *L. lavandulifolia*. The HPTLC analysis confirms the quantitative determination of bioactive metabolites in the plant. Quercetin is a flavonoid whereas the syringic acid is a phenolic component both shows the anti-oxidant property and in *L. lavandulifolia* the percentage of quercetin, as well as the syringic acid, is present in a good amount, which was not reported in previous literature. These types of studies on natural products are designed to establish the importance of medicinal properties of plants by correlating with the traditional uses and scientific knowledge to discover the active potential drug which will utilize by the pharmaceutical industries.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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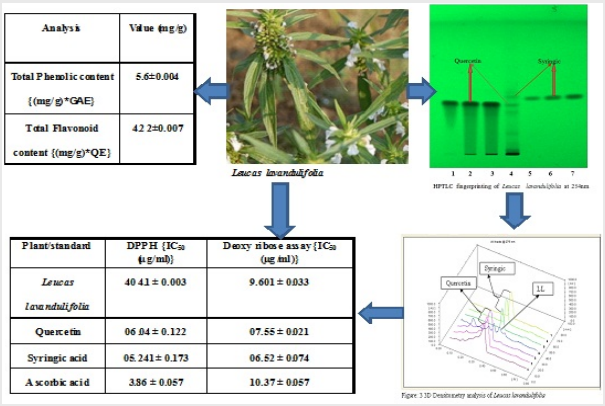
PICTORIAL ABSTRACT



SUMMARY

- Phytochemicals are found within the standard limit of Ayurvedic Pharmacopoea of India.
- Identification of phenolic bioactive compounds.
- Development of HPTLC method for the quantification of pharmacologically active metabolite *i.e.* quercetin and syringic acid.
- Promising *in-vitro* antioxidant potential were observed in the *L. lavandulifolia.*

About Authors

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