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Exploring the Anti-cancer Effect of Ethanol Extract of Jamaican Cherry (*Muntingia calabura* L.) Leaves on CT26 Colorectal Cancer Cells through WNT Signaling

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ABSTRACT

The prevalence rate of mortality in colorectal cancer is comparatively elevated. A standard or effective treatment has not been developed to resolve this issue. Utilizing *Muntingia calabura,* which is known to contain rutin with anti-cancer properties, is one of the strategies that can be implemented. The present study focuses on the potential therapeutic effects of *M. calabura* as an anti-cancer agent in colorectal cancer. Specifically, this study investigates the anti-cancer properties of rutin against colorectal cancer. The results of this study showed that the ethanolic extraction of *M. calabura* contained approximately 0.727 mg/mL of rutin. Additionally, the cytotoxicity assessment revealed that the rutin fraction was moderately cytotoxic, with a value of 70.808 µg/mL. The results of molecular docking experiments demonstrated a high degree of accuracy in predicting the interaction between rutin and β-catenin. Furthermore, the quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay revealed a significant reduction in the expression of the β-catenin gene within the cells exposed to the extract. In conclusion, *M. calabura* extract exhibits anti-cancer properties by influencing the WNT signaling pathway in CT26 cells.
Keywords: Anti-cancer; Colorectal cancer; *M. calabura*; Rutin

INTRODUCTION

The prevalence of colorectal cancer has shown an annual rise in occurrence. Notably, there has been a significant increase in the number of reported cases of this disease, with figures going from 1.8 million cases (resulting in 881,000 deaths) in 2018 to 1.9 million new cases (leading to 935,173 fatalities) in 2019 (Baidoun et al., 2021). The epidemiological characteristics of colorectal cancer in Indonesia closely align with global statistics, as it is fourth in cancer-related mortality. Specifically, there have been a total of 234,511 reported deaths attributed to colorectal cancer in Indonesia (Globocan, 2020).

Surgical intervention is typically limited to sites near the colorectal region, such as the liver. In the context of cancer treatment, it is important to note that chemotherapy necessitates a consistent administration of drugs to achieve desired therapeutic outcomes. However, it is worth acknowledging that this approach may give rise to undesirable adverse consequences (Clark et al., 2022).

Cancer cell proliferation is an essential phase in the malignancy process of colorectal cancer. The proliferation process can be attributed to mutations in the adenomatous polyposis coli (APC) gene, resulting in the intricate disruption of negative modulators, including casein kinase 1 (CK1), GSK3 beta, AXIN, β-catenin, and APC. The outcome of this intricate destruction process is a build-up of β-catenin (Egbuna, 2023). Furthermore, the activation of RNA polymerase occurs continually to initiate the transcription process of the genes that encode MYC and cyclin D1 (Fares, 2020). This activation is facilitated by synthesizing transcription factors with T-cell factor (TCF), which is brought about by the build-up of β-catenin. This results in a notable escalation in the proliferation of colorectal cancer cells, as reported by Zhao et al. (2022).

Based on the explanation above, it can be observed that the incidence of mortality resulting from colorectal cancer is comparatively elevated. Over the past few decades, chemically synthesized drugs have not significantly improved the survival rate (Choudhari, 2020). Surgical removal is limited to specific circumstances, whereas chemotherapy-targeted therapy is ineffective in addressing malignancies resulting from APC mutations. Therefore, to increase the effectiveness of current cancer treatment, new strategies and novel

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chemoprevention agents are needed to complement current cancer therapies. Additional investigation is warranted to mitigate the influence of APC gene alterations on colorectal cancer cells, with one potential avenue being the utilization of a phytopharmaceutical strategy (Sriwidyani, 2013).

This study used *Muntingia calabura* as an anti-colorectal cancer agent in a phytopharmaceutical approach (Nasir, 2017). Based on data from IUCN (2023), *M. calabura* is the least abundant plant in Indonesia, and its potential is not utilized optimally. Even though *M. calabura* has several pharmacological effects that have been proven beneficial for the medical world in Indonesia, *Muntingia calabura's* medicinal value is not well documented in Indonesia and is considered a neglected plant (Mahmood, 2014). One of its pharmacological effects is an anti-cancer agent that inhibits the Wnt signaling pathway. According to Lin et al. (2018), *M. calabura* has 4 main flavonoids: epicatechin, rutin, diosmin, and luteolin. Of these flavonoids, rutin has the best binding interaction with β-catenin protein based on its binding affinity from in silico prediction (Egbuna, 2023). This indicates its potential as an anti-cancer agent that inhibits the Wnt signaling pathway better than epicatechin, diosmin, and luteolin (Zhao et al., 2022). This finding is consistent with the results of prior scholarly investigations, which have demonstrated that the methanol extract derived from *M. calabura* can initiate apoptosis in colorectal cancer cells at an early stage (Jisha et al., 2020). In addition to these attributes, *M. calabura* has the qualities of being readily accessible, exhibiting longevity, exhibiting non-seasonal growth patterns, and displaying adaptability to various soil types (Ansori, 2021).

MATERIALS AND METHOD

Extraction of *Muntingia calabura*

Jamaican cherry (*Muntingia calabura*) was harvested independently in June in Samigaluh District, Kulon Progo Regency, Special Region of Yogyakarta. The leaf segment utilized for this study was the third segment of the adult leaf, originating from the distal end of the stem (Pandey et al., 2021). The collected leaves were determined in the Department of Pharmaceutical Biology, Faculty of Pharmacy, UGM, with acceptance number 9.18.7/UN1/FFA.2/BF/PT/2023, and the results confirmed that the leaves collected were indeed *M. calabura* leaves. It was then oven-dried at 45 °C for 72 hours before being pulverized with a simplicial grinder. The extraction process involved the maceration of powdered simplicia derived from dried leaves of *Muntingia calabura* (Al-Aadily, 2022). A quantity of 100 grams of powdered dry *Muntingia calabura* was subjected to extraction using 1000 milliliters of ethanol (General Labora, Yogyakarta, Indonesia) with a concentration of 70% (v/v) in a ratio of 1:10 (w/v) for 72 hours, with regular stirring every 8 hours (Pohanka, 2016). The mixture was stirred at regular intervals of 8 hours. The extract underwent filtration using a Buchner filter and subsequent concentration through the application of a water bath set at a temperature of 40 °C (Buhian, 2016).

Phytochemical Characterization

Along with a micropipette, 5 µL of a thick extract with a concentration of 20,000 µg/mL was added to the stationary phase of silica gel F254 (1.05554.0001, USA). Then, it was eluted with a mixture solvent of butanol (1.01990.1000, USA), acetic acid (1.00063.2500, USA), and water in a ratio of 3:1:1. Samples were compared with a rutin reference of 10% and detected under UV light at a wavelength of 254 nm (Perk, 2014). The spots on the plate glowed greenish yellow, and then the Rf value was calculated. The TLC results were then quantified using the densitometry method via a TLC scanner based on the equation of the area under the standard curve with a rutin comparison (Zebua et al., 2019). The yield of the extract obtained was calculated using the following formula:

$$\% \text{ yield} = \frac{Extract\ weight}{simplicity\ weight} \times\ 100\%$$

Cell Cytotoxicity Activity Test

Preparation media of CT26 cells with a density of 1×10^4 cells/well was put into a microplate (96-well plate). Media preparation for MTT assay consisted of RPMI (11875093, USA), penicillin-streptomycin (15140122, USA), fungizone (15290-018, USA), and FBS (F7524-500ML, USA). Microplates were incubated for 24 hours in a CO2 incubator at 37 °C. Following this, 100 µl of the test sample (1000 µg/mL; 750 µg/mL; 375 µg/mL; 250 µg/mL; 15.625 µg/mL; 7.8125 µg/mL; 3.90625 µg/mL; 1.953125 µg/mL; 0.976563 µg/mL) was added to the microplate. The microplate was incubated again for 24 hours in a CO2 incubator (Sufian, 2012). Subsequently, cells were washed with PBS (10010-023, USA). Then, 100 µl of new complete media and 10 µl of 0.5% MTT reagent (T0793, USA) were added to the microplate. It was then incubated for 3 hours in a CO_2 incubator at 37 °C. The microplate that had been incubated was added to 100 µl of DMSO 0.5% (1.02952.2500, USA). The results were read using an ELISA reader at a wavelength of 550 nm. The obtained absorbance (optical density) data was

then calculated to determine the IC_{50}value (Arifah, 2015). The percent cell cytotoxicity was calculated using the following equation:

$$\text{Cell cytotoxicity} = \frac{Treatment\ absorbance - Media\ control\ absorbance}{Cell\ control\ absorbance - Media\ control\ absorbance} \times 100\%$$

Data processing used one-way ANOVA using Microsoft Excel software to evaluate the IC_{50} value.

Compound Analysis by Molecular Docking

Compound analysis was done using molecular docking with the MOE. To carry out molecular docking, it is necessary to have data in the form of protein structures obtained from two databases. The β-catenin receptor structure (PDB ID: 6TFB) was retrieved from the Protein Data Bank (https://www.rcsb.org/), while the ligand rutin, identified through the phytochemical characterization of Muntingia calabura extracts, was obtained from the PubChem database. Then, information about the binding of β-catenin to the active compound rutin of *M. calabura* extract was downloaded. The aim was to identify the compounds that might interact with one another the most effective way possible (Ridwan, 2023). The molecular docking results were analyzed with MOE to determine the GDP ID value for the RMSD value and ΔG value (kcal/mol) analysis.

Real-Time Quantitative Reverse Transcription PCR Analysis

Total RNA was extracted using Trizol (Smobio, Taiwan) from the RNA extraction kit (Smobio, Taiwan). The first strand of cDNA was prepared from an RNA template (25 ng) using the oligo(dT)18 primer and the Power cDNA synthesis kit (Songjaeng, 2022). Reverse transcriptase was carried out at 42 °C for 50 minutes, followed by 70 °C for 15 minutes. qRT-PCR (ABi 7500 FAST) used the Power SYBR Green PCR master mix (Smobio, Taiwan) and the TwoStep reverse transcription PCR system. All data were normalized to β-actin mRNA (Kee et al., 2017). The DNA primer sequences for this analysis are described in Table I.

All data were collected from at least three independent experiments. Data sets were analyzed using Microsoft Excel (Microsoft Office Home and Student 2021, USA). In this study, we used singleplex qRT-PCR assay to detect and quantify CT26. The qRT-PCR reading results were analyzed using Bio-Rad CFX Manager software. Quantification results were subsequently analyzed for relative expression using the Livak method (Livak & Schmittgen, 2021).

$\Delta Ct = CtGen - CtRef$
$\Delta\Delta Ct = \Delta CtTreatment - \Delta CControl$
Relative expression = $2^{-\Delta\Delta Ct}$

Analysis Method

Data from quantification results obtained from each group of cells were then analyzed statistically using the t-test to compare each treatment group with the control group. The ANOVA test ($P<0.05$) was employed to compare treatment groups with data confidence of 95%. Data are presented as mean ± standard deviation (SD), with GraphPad Prism 9.00 computer program.

RESULTS

Extraction of *Muntingia calabura*

The obtained extraction results were subjected to evaporation, producing 10.3 grams of a concentrated extract. The yield value of this extraction process was determined to be 10.3%.

Identification of Rutin Compounds in *Muntingia calabura* Extract

The qualitative analysis was conducted using thin layer chromatography (TLC). Upon exposed to UV light at a wavelength of 366 nm, greenish-yellow spots were observed in the sample at an Rf value of 0.70. Subsequently, a comparative retention factor (Rf) value of 0.68 was achieved on the thin-layer chromatography (TLC) plate. This indicates the presence of rutin in the *Muntingia calabura* leaf extract.

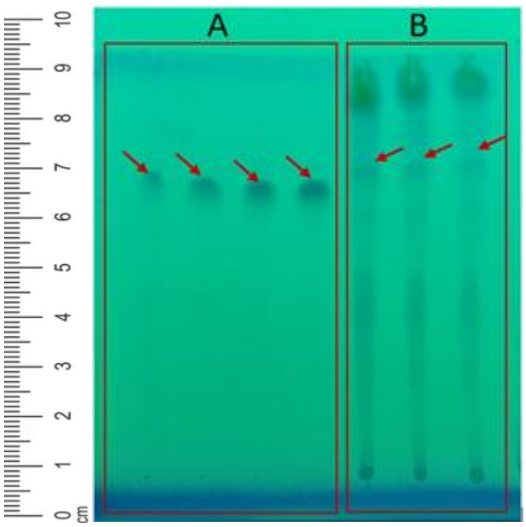

Figure 1. Results of TLC test on ethanol extract of *Muntingia calabura* leaves

Description: Results of rutin comparative TLC test under 254 nm UV light (A), TLC test results of *Muntingia calabura* ethanol extract under 254 nm UV light (B)

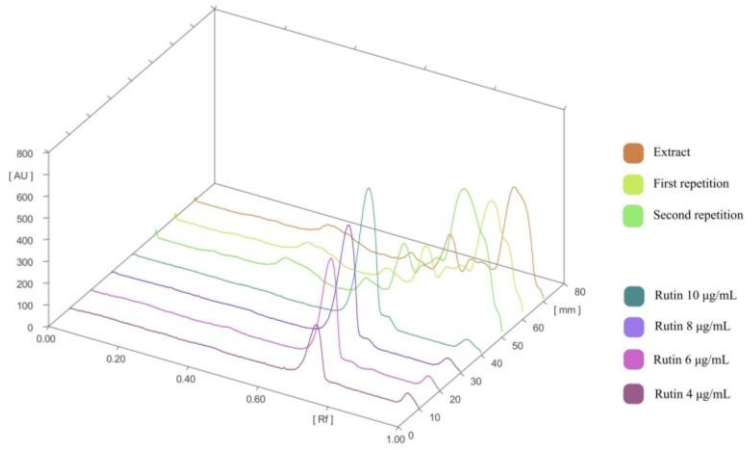

Figure 2. Results of densitometry test on ethanol extract of *Muntingia calabura* leaves

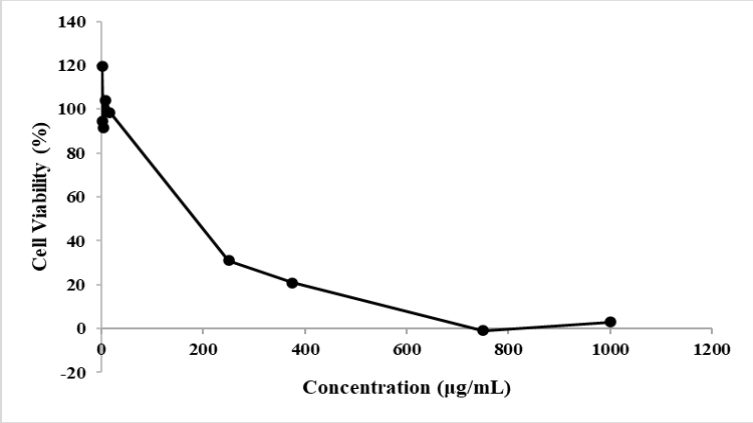

Figure 3. Cell concentration with % cell viability. It can be seen that the higher the concentration, the lower the cell viability.

Table I. Primer sequences and housekeeping gene sequences

Gen	Forward (5′-3′)	Reverse (5′-3′)
β-catenin	GTTCGCCTTCATTATGGACTGCC	ATAGCACCCTGTTCCCGCAAAG
β-actin murine	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

In the quantitative test utilizing densitometry, a regression equation of y = 3134x - 1815.6 was derived from the standard concentration series of 4, 6, 8, and 10 µg. The coefficient of determination (R^2) for this regression equation was determined to be 0.9663. In this study, the sample was subjected to replication three times, utilizing a spot volume of 5 µL. The resulting average concentration of rutin in the extract derived from *Muntingia calabura* leaves was 0.727 mg/mL.

Effect of *Muntingia calabura* Extract on Cell Cytotoxicity

The cytotoxicity of *Muntingia calabura* extract on cells was evaluated using the MTT Assay. The results yielded an IC_{50} value of 70.808 µg/mL, indicating that a concentration of *Muntingia calabura* extract at 70.808 µg/mL was required to induce cell death in 50% of the cancer cell population. The IC_{50} value was determined using the linear regression equation y = -39.355x + 122.81, where y represents the response variable, and x is the concentration of the compound. The coefficient of determination (R^2) for this equation was 0.932, indicating a strong correlation between the concentration of the compound and the response variable. The IC_{50} value fell within the range of 21–200 µg/mL, as reported by Canga (2022), placing it into the moderate cytotoxic or moderately active classification.

An inverse relationship was observed between the concentration of *Muntingia calabura* extract and the viability of cells, where an increase

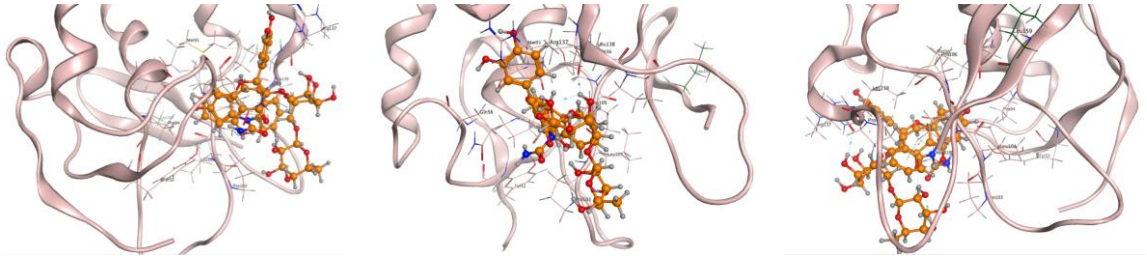

Figure 4. The affinity of the rutin ligand (orange) with the PDB 6TFB protein in molecular Docking

Table II. Results of rutin RMSD molecular docking values for GDP 6TFB

Gene	6TFB
ΔG (kcal/mol)	-15.2271
RMSD	1.4286

in extract concentration resulted in a decrease in cell survival. This observation indicates that the extract's concentration impacts the viability of cells (Onyoh, 2019). This result implies that an increase in the concentration of *M. calabura* is associated with a decrease in the viability of cancer cells or an increase in cell inhibitory action (Hapach, 2019). The findings of this study demonstrate the inhibitory effects of *Muntingia calabura* extract on CT26 colorectal cancer cells (Castle et al., 2014).

Affinity Analysis of Compounds with Ligands

The molecular docking of β-catenin was performed on the musculus utilizing the Protein Data Bank (PDB) code 6TFB and rutin ligands, employing the MOE program. Molecular docking experiments were conducted utilizing an HP laptop 14-em0xx system, which belongs to the x64-based PC category, from August 14th to August 19th, 2023. The findings from the molecular docking analysis revealed that the ligand exhibited binding affinity towards specific amino acids, namely Pro (105), Met (91), Leu (138), Gln (56), and Arg (137). The resulting root-mean-square deviation (RMSD) value was 1.4286, while the corresponding change in free energy (ΔG) was -15.2271 kcal/mol.

The root mean square deviation (RMSD) value quantifies the degree of errors that arise during docking. A desirable RMSD value is often below 2, indicating a lower level of deviation. A root means square deviation (RMSD) value of below 2, when comparing the Protein Data Bank (PDB) entry 6TFB with rutin, signifies high accuracy and precision in the docking conformation outcomes. This indicates that rutin exhibits a high affinity for β-catenin, suggesting that rutin possesses significant anti-proliferation properties.

Analysis of Downstream Gene Expression in the WNT Pathway

Four distinct treatments were employed to investigate the quantitative real-time polymerase chain reaction (qRT-PCR). These treatments encompassed control cells, the housekeeping gene β-actin murine, and two groups of three extract concentrations, specifically 50 µg/mL and 12.5 µg/mL. Subsequently, the primers were optimized at a temperature ranging from 56 to 58 °C. The RNA extraction process was carried out using the Favorgen RNA kit, allowing for the extraction of RNA up to 2000 ng. Additionally, complementary DNA (ccDNA) was prepared at a concentration of

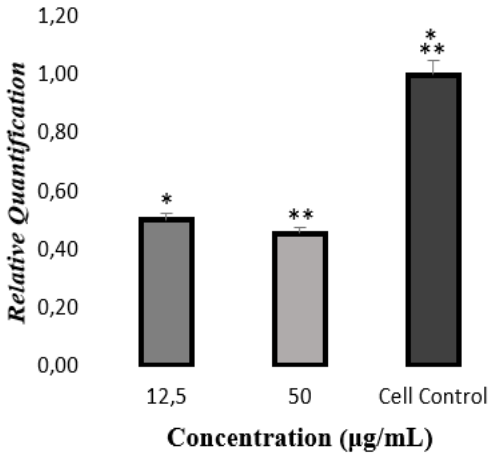

Figure 5. Diagram of relative quantification value analysis. The efficiency of *M.calabura* genome detection. Asterisks * show that the control cells, which were not given the extract, were higher. Meanwhile, the asterisks * and ** indicate positive downstream effects, where an increase in the concentration led to a decrease in the cell proliferation potential.**

up to 25 ng/µL. The findings of quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrated a relative quantification (Rq) value of 1 in the control cells. In contrast, the treated cells exhibited an Rq value of less than 1. These findings suggest that the extract led to a downregulation of β-catenin gene expression in the exposed cells.

DISCUSSION

The present study on the anti-colon cancer potential of ethanol extracts of *Muntingia calabura* and the association between anti-proliferation activity as a part of the mechanisms of anticancer activity. The proliferation of CT26 colorectal cancer cells is driven by mutations in the adenomatous polyposis coli (APC) gene, leading to complex disruptions of negative modulators, such as casein kinase 1 (CK1), GSK3 beta, AXIN, β-catenin, and APC. This disruption culminates in the accumulation of β-catenin (Egbuna, 2023). The accumulation of β-catenin triggers the proliferation of colorectal cancer. *Muntingia calabura* can inhibit this proliferation by binding to β-catenin and preventing the downregulation activity of β-catenin.

The ability of ethanol extracts of *Muntingia calabura* to anti-proliferation might also be attributed partly to the extract's high binding affinity from molecular docking analysis between rutin and β-catenin. This high binding affinity is conducted to a more potent inhibitor, and it can effectively block the protein's function at lower concentrations. The findings from the molecular docking analysis revealed that the ligand exhibited binding affinity towards specific amino acids, namely Pro (105), Met (91), Leu (138), Gln (56), and Arg (137). Affinity analysis utilizing a molecular docking approach revealed that the root-mean-square deviation (RMSD) value was 1.4286, and the calculation of the associated change in free energy (ΔG) resulted in a value of -15.2271 kcal/mol, indicating a high binding affinity between rutin and β-catenin.

Rutin was detected in ethanol extracts of *Muntingia calabura* through qualitative analysis using thin-layer chromatography (TLC). These extracts exhibited cytotoxicity, with an IC_{50} value of 70.808 µg/mL, signifying the concentration necessary to induce cell death in 50% of the cancer cell population. As reported by Canga (2022), this IC_{50} value, within the range of 21–200 µg/mL, indicates that the extract is moderately cytotoxic or active. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) demonstrated downstream gene expression within the Wnt pathway. The efficiency of *Muntingia calabura* genomic detection indicates that the control cells, which were not exposed to the extract, exhibited higher proliferation potential. Conversely, cells treated with *Muntingia calabura* at concentrations of 12.5 µg/mL and 50 µg/mL showed positive downstream effects. Notably, higher concentrations were associated with a greater reduction in cell proliferation potential. These findings suggest that ethanol extracts of *Munitingia calabura* demonstrate an anticarcinogenic activity against CT26 colorectal cancer in BALB/c mice through the synergistic action of these chemicals.

CONCLUSION

This research has shown that the rutin fraction of the ethanol extract of *M. calabura* can inhibit the proliferation of CT26 colorectal cancer cells via the WNT signaling pathway. Nevertheless, alternative testing yielded contradictory results. The cytotoxicity of *M. calabura* extract on CT26 cells was moderate, as shown by the IC50 value of 70.808 µg/mL. Through the utilization of molecular docking techniques, it was determined that there was a notable affinity between rutin and the protein denoted as PDB 6TFB. This finding suggests that rutin can function as an agent with anti-proliferation properties. Furthermore, the findings from the examination of β-catenin gene expression indicated a reduction in cells subjected to *M. calabura* extract.

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