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Comparative Evaluation of the Antibacterial Activity of Alcoholic Extracts from *Ziziphus spina-christi* and *Retama raetam* Against Selected Gram-Positive and Gram-Negative Pathogens Using the Disc Diffusion Assay

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Abstract

Background: The increasing prevalence of antimicrobial resistance among clinically relevant bacterial pathogens underscores the need for effective plant-derived antibacterial agents. Ziziphus spina-christi (Sidr) and Retama raetam are traditionally used medicinal plants known to contain phenolic and flavonoid compounds with potential antimicrobial properties. Aim: This study aimed to comparatively evaluate the antibacterial activity of ethanolic extracts of Z. spina-christi and R. raetam against Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae using the standardized disc diffusion method. Methodology: Bacterial inocula standardized to 0.5 McFarland were tested using the Kirby–Bauer disc diffusion assay on Mueller–Hinton agar. Three defined concentrations of each plant extract were applied to sterile discs, along with solvent controls and a reference antibiotic. After incubation at 37°C for 18–24 h, the inhibition zones were measured in millimeters. Data were analyzed using one-way analysis of variance (ANOVA) and appropriate post hoc tests. Results: A clear concentration-dependent antibacterial effect was noted. Higher extract concentrations produced significantly larger inhibition zones for all bacterial species. Z. spina-christi extract demonstrated greater antibacterial potency than R. raetam, particularly against S. aureus, while both extracts inhibited E. coli and Klebsiella to varying degrees. Conclusion: The ethanolic extracts of Z. spina-christi and R. raetam exhibited promising antibacterial activity, supporting their potential use as complementary natural antibacterial agents. Further MIC/MBC studies and phytochemical analysis are recommended.

Keywords:Ziziphus spina-christi, Retama raetam, antibacterial activity, disc diffusion assay, inhibition zone, Gram-positive bacteria, Gram-negative bacteria.

Introduction

Antimicrobial resistance (AMR) poses a growing threat to global public health, diminishing the efficacy of conventional antibiotics and increasing the need for alternative therapeutic agents (Hussein et al., 2023). Medicinal plants are an important source of bioactive secondary metabolites, including alkaloids, phenolics, and flavonoids, which exhibit promising antibacterial properties (El-Shahir et al., 2022).

Ziziphus spina-christi (Sidr) has a long history of ethnomedicinal use, supported by modern research confirming its high polyphenol and flavonoid content with antimicrobial activity (Badr et al., 2020).

Similarly, Retama raetam is a xerophytic species rich in phytochemicals, such as quinolizidine alkaloids, flavonoids, and phenolic compounds, and has demonstrated antibacterial and antioxidant activities (Al-Onazi et al., 2021; Soriano et al., 2022).

Among these plants, Ziziphus spina-christi (commonly known as Sidr) holds a distinguished place in ethnopharmacology in the Middle East and North Africa. Its leaves, fruits, and bark are widely used to treat infections, inflammation, and wounds **(Temerk 2017).** Modern phytochemical analyses have confirmed that Z. spina-christi contains a variety of polyphenols, flavonoids, and triterpenoids with potent antioxidant and antimicrobial activities **(El-Shahir et al. 2022).** In vitro studies have demonstrated significant inhibition zones against gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*, and gram-negative strains, such as *Escherichia coli* and *Pseudomonas aeruginosa* **(Badr et al., 2020)**. Additionally, nanoformulation approaches, such as silver-cored nanosuspensions loaded with Z. spina-christi extract, have enhanced antibacterial efficacy and helped overcome multidrug resistance **(Hussein *et al.*, 2023)**. Despite these promising findings, few studies have systematically compared the antibacterial spectra of alcoholic extracts across multiple bacterial classes or explored their potential roles as biocontrol agents in plants.

Similarly, Retama raetam (Forssk.) Webb & Berthel., a xerophytic shrub belonging to the Fabaceae family, is known for its rich phytochemical profile and allelopathic activity (insert citation). Native to the arid and semi-arid regions of the Mediterranean Basin and Arabian Peninsula, this plant has been reported to contain diverse secondary metabolites, including quinolizidine alkaloids, flavonoids, phenolics, and essential oils **(Al-Onazi et al., 2021).** Several studies have demonstrated the antibacterial, antioxidant, and insecticidal activities of R. raetam extracts, supporting its potential as a natural antimicrobial and biopesticide **(Soriano et al., 2022).** Notably, advanced nanophytosome formulations combining R. raetam extracts with antibiotics, such as colistin, have improved antibacterial and neuroprotective effects, further emphasizing their pharmacological and agricultural relevance **(Elsabrouty et al., 2024).** However, existing data are fragmented, and there is a scarcity of comparative studies investigating the antibacterial and biocontrol activities of R. raetam alongside other medicinal plants using standardized methodologies

Plant-based biocontrol agents are increasingly viewed as sustainable alternatives to synthetic agrochemicals, offering lower toxicity and reduced risk of environmental contamination **(Soriano et al., 2022).** However, most prior research on Z. spina-christi and R. raetam has focused primarily on their antimicrobial or antioxidant properties in vitro, without extending to plant-pathogen biocontrol models. Furthermore, studies often vary in extraction solvents, bacterial strains tested, and assay conditions, complicating cross-comparison and weakening the generalizability of the results **(Badr**

et al., 2020; El-Shahir et al., 2022). Addressing these inconsistencies requires a unified experimental framework to evaluate the activity spectrum, potency, and potential synergistic interactions of the extracts against both clinical and phytopathogenic bacteria.

Despite these insights, comparative studies using standardized experimental methods are limited. Therefore, this study aimed to systematically evaluate and compare the antibacterial activities of ethanolic extracts from these two plants using a standardized disc diffusion assay.

Materials and Methods

Study design

A laboratory-based experimental design was employed to assess inhibition zone diameters produced by alcoholic extracts from Ziziphus spina-christi and Retama raetam against selected Gram-positive and Gram-negative bacteria. This study followed a completely randomized design, testing different extract concentrations against defined bacterial strains, with all treatments performed in triplicate.

Bacterial strains and inoculum standardization

Three bacterial species were tested: *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The strains were obtained from the bacteriology laboratory collection (clinical isolates previously identified by standard biochemical tests) and maintained on nutrient agar slants at 4 °C until use for the experiments. For each experiment, a fresh overnight culture on Mueller–Hinton agar (MHA) was prepared at 37 °C for 18–24 h. Bacterial suspensions were adjusted to a turbidity equivalent to the 0.5 McFarland standard (approximately 1×10^8 CFU/mL) by visual comparison against a commercial 0.5 McFarland tube and verified when possible by optical density measurement at 625 nm.

Plant material and preparation of alcoholic extracts

The leaves of Z spina-Christi and the aerial parts of R raetam were collected from local markets, morphologically authenticated, and thoroughly washed with sterile distilled water. The plant material was shade-dried at room temperature and then oven-dried at 40–45 °C to a constant weight. The dried samples were ground into a fine powder and stored in airtight containers at 4 °C until extraction. For each plant, 20 g of powder was macerated with 200 mL of 96% ethanol for 24–48 h at room

temperature, with intermittent shaking. The mixtures were filtered sequentially through sterile gauze and Whatman No. 1 filter paper, respectively. The filtrates were concentrated under reduced pressure using a rotary evaporator at 40–45 °C, and the crude extracts were dried to a constant weight, weighed, and stored at 4 °C in amber vials. Working solutions were prepared in sterile ethanol and further diluted with sterile distilled water to obtain the desired test concentrations.100%%25 ،%50 ،

Culture media preparation

Mueller–Hinton agar (MHA) was used for agar diffusion assays according to CLSI recommendations. Commercially dehydrated MHA was prepared by dissolving 38 g in 1 L of distilled water, heating with gentle stirring until complete dissolution, and autoclaving at 121 °C for 15 min. After autoclaving, the medium was allowed to cool to 45–50 °C and poured into sterile Petri dishes (approximately 20–25 mL per plate) under aseptic conditions to obtain a uniform depth of 4 mm of medium. The plates were left to solidify at room temperature and dried briefly before inoculation.

Antibacterial sensitivity testing (disc diffusion assay)

The antibacterial activity of the plant extracts was assessed using the standard Kirby–Bauer disc diffusion method on MHA. Sterile cotton swabs were dipped into a 0.5 McFarland bacterial suspension, excess fluid was removed by pressing against the tube wall, and the suspension was evenly spread over the entire surface of the MHA plates in three directions to obtain a uniform lawn. Sterile 6 mm Whatman filter paper discs were impregnated with 20 µL of each extract concentration and allowed to dry briefly under sterile conditions to avoid over-wetting. Discs were then gently placed on the inoculated agar surface using sterile forceps, ensuring adequate spacing between the discs. For each bacterial strain and plant extract, three concentrations were tested, and each treatment was performed in triplicate.

Controls

Negative control discs were loaded with the highest volume of solvent used (ethanol diluted in sterile distilled water) without the plant extract to assess the solvent effects. Positive control discs contained a standard antibiotic (e.g., gentamicin 10 µg or ampicillin 10 µg) for comparison with a clinically relevant reference drug. All control discs were placed on the same plates as the test discs, whenever possible.

Incubation and measurement of inhibition zones

The inoculated plates with the applied discs were incubated at 37 °C for 18–24 h in an inverted position. After incubation, the antibacterial effect was evaluated by measuring the diameter of the clear inhibition zones (including the disc) around each disc in millimeters using a digital or Vernier caliper for increased accuracy. For each treatment, the mean inhibition zone and standard deviation were calculated from three independent replicate plates per treatment.

Data analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA to compare means among different concentrations, bacterial species, and extracts, followed by appropriate post hoc tests (Tukey or Games–Howell) at a significance level of $p < 0.05$.

RESULS

Descriptive Statistics of Bacterial Counts

The descriptive statistics (Table 1) indicated a clear inverse relationship between the extract concentration and bacterial growth inhibition. While the original data presented 'Bacterial Counts', these values are interpreted here as a measure of relative growth/resistance. The highest 'counts' (indicating the lowest inhibition) were observed at the lowest extract concentration (25%), reinforcing the concentration-dependent effect. *Klebsiella* exhibited the highest mean 'counts' (lowest susceptibility), suggesting greater resilience compared to S. aureus and *E. coli."*

Table1 **Mean Inhibition Zone Diameters (mm) by Bacterial Species and Extract Concentration**

Types of bacteria	N	Mean ± SD	95% CI
Staphylococcus aureus	18	15.60 ± 2.90	14.16 – 17.04
Escherichia coli	18	18.74 ± 3.16	17.16 – 20.31
Klebsiella	18	20.07 ± 3.31	18.43 – 21.72

Table2. **Comparative Mean Inhibition Zone Diameters (mm) for Z. spina-Christi (Sidra) and R. raetam (Rhythm) Extracts**

Extract type	N	Mean ± SD	95% Confidence Interval	
LL	*UL*			
Sidra	27	16.85 ± 3.64	15.411	18.294
Rhythm	27	19.42 ± 3.14	18.179	20.66

Table 2 presents a comparative analysis of the mean scores between the Sidra and Rhythm groups, with a sample size (N) of 27 participants in each group. The Sidra group had a mean score of 16.85

(standard = 3.64), whereas the Rhythm group had a higher mean score of 19.42 (SD = 3.14). The 95% confidence intervals for the Sidra group ranged from 15.411 to 18.294, indicating the range within which the true mean likely falls, whereas the Rhythm group's confidence interval spanned from 18.179 to 20.660. Notably, the significance value (Sig) for the Sidra group was 0.008, suggesting a statistically significant difference between groups. This indicates that the intervention associated with the Rhythm group may have had a more pronounced effect than that of the Sidra group, highlighting important implications for future research and practice

Table 3. Tukey's Honest Significant Difference Multiple Comparisons post hoc test.

Bacterial Type	Bacterial Type	Mean Difference	Std. Error	Sig.	95% CI of the Difference	
LL	***UL***					
Staphylococcus aureus	*Escherichia coil*	-3.13*	1.044	0.011	- 0.615	-3.134
Klebsielle		-4.47*	1.044	> 0.001	- 6.992	-1.953
Escherichia coil	*Staphylococcus aureus*	3.13*	1.044	0.011	0.615	5.654
Klebsielle		-1.34	1.044	0.412	-3.858	1.181
Klebsielle	*Staphylococcus aureus*	4.47*	1.044	> 0.001	1.953	6.992
Escherichia coil		1.34	1.044	0.412	-1.181	3.858

"The Tukey HSD post hoc test (Table 3) revealed significant differences in antibacterial efficacy among the three bacterial species (with respect to the mean inhibition zone diameters). Specifically, *S. aureus* showed significantly higher susceptibility (larger inhibition zones) compared to *E. coli* and *Klebsiella* ($P < 0.05$), confirming that the extracts' activity is species-dependent."

Effect of Extract Type and Concentration

- Games-Howell post hoc tests, used due to unequal variances, demonstrated significant differences in bacterial resistance patterns across species and extract concentrations.
- The strongest contrasts were observed between Sidra 100% *Staphylococcus* aureus and Sidra 25% *Escherichia coli*, indicating varying resistance depending on the bacterial strain and concentration.
- These findings underscore the importance of considering bacterial species and extract concentration in antimicrobial strategies.

Table 4. Independent Sample t-test Results Comparing Bacterial Counts Between Sidra and Rhythm Extracts

Test	t	df	Mean Difference	Std. Error	Sig.	95% CI of the Difference	
LL	***UL***						
Independent Samples t-test	**-2.774**	**52**	**-2.567**	**0.925**	**0.008***	**-4.423**	**-0.71**

***. The mean difference is significant at the 0.05 level**

Multiple Comparisons for Resistance Profiles

- Games-Howell post hoc tests, used due to unequal variances, demonstrated significant differences in bacterial resistance patterns across species and extract concentrations.
- The strongest contrasts were observed between Sidra 100% *Staphylococcus* aureus and Sidra 25% *Escherichia coli*, indicating varying resistance depending on the bacterial strain and concentration.
- These findings underscore the importance of considering bacterial species and extract concentration in antimicrobial strategies.

Table 5. Multiple Comparisons of Bacterial Count Using Games-Howell

Group I	Group II	Mean Difference (I-J)	Significant Differences (Games-Howell, p<0.05)
Sidra 100% Staphylococcus	Sidra 25% Escherichia coil	- 8.597*	0.048
Sidra 100% Staphylococcus	Sidra 25% Klebsielle	-9.88*	0.04
Sidra 100% Staphylococcus	Rhythm 25% Klebsielle	-11.557*	0.014
Sidra 100% Escherichia coil	Rhythm 25% Klebsielle	-9.36*	0.034
Sidra 25% Escherichia coil	Rhythm 100% Staphylococcus	6.64*	0.018
Rhythm 100% Staphylococcus	Rhythm 25% Escherichia coil	-8.023*	
Rhythm 100% Staphylococcus	Rhythm 25% Klebsielle	-9.6*	0.043
Rhythm 100% Klebsielle	Rhythm 100% Staphylococcus	6.507*	0.011

Analysis of Variance (ANOVA) Results

- One-way ANOVA revealed significant differences in bacterial counts among the three species ($p < 0.001$).
- Post hoc Tukey tests indicated that *Staphylococcus aureus* had significantly lower counts than *Escherichia coli* and *Klebsiella*, whereas no significant difference existed between *E. coli* and *Klebsiella*.
- ANOVA also showed statistically significant differences among the concentration levels, confirming that the bacterial counts increased as the extract concentration decreased**.**

Table 6. Summary of ANOVA Results Showing Differences in Bacterial Counts Among Concentration Levels

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
Between Groups	225.154	2	112.577	12.358	> 0.001
Within Groups	464.584	51	9.109		
			Total	689.738	53

Interactive Effects Illustrated in Figure 1

- Figure 1 shows the interactive effect of extract type and concentration on the mean bacterial counts (CFU/mL) for the three species.
- Increasing the extract concentration was correlated with decreased bacterial counts.
- Sidra extract was most effective against S. aureus, whereas Rhythm was more effective against *E. coli*.

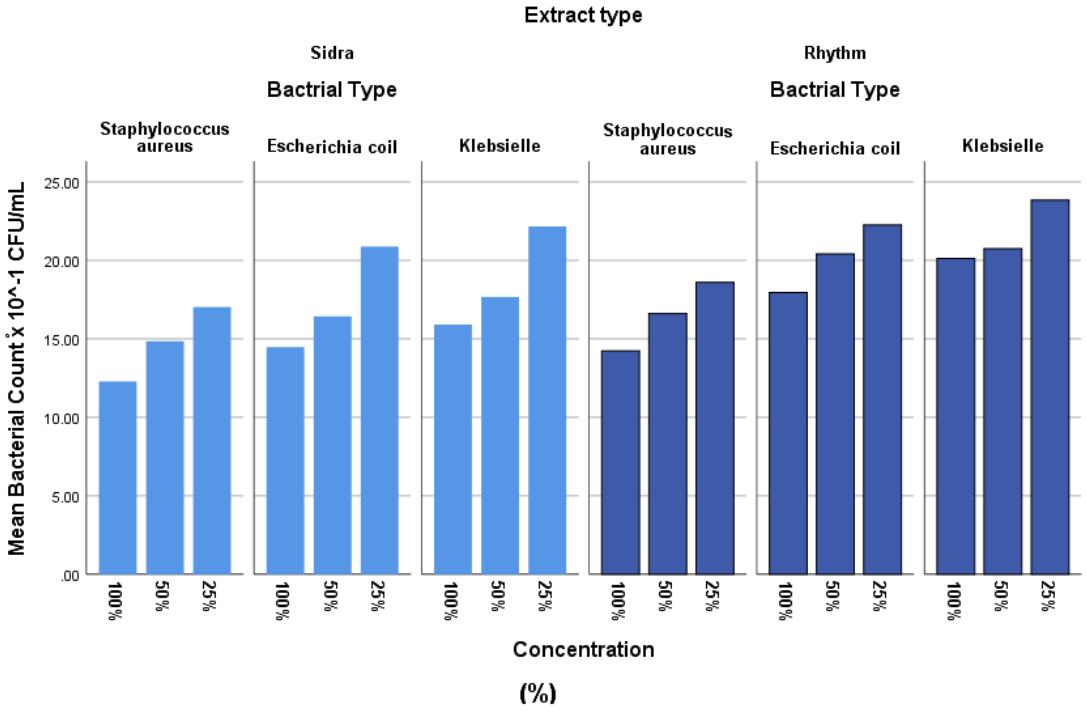

Figure (1) The Interactive Effect of Extract Type and Concentration on Mean Bacterial Count (CFU/mL)

Discussion

This study demonstrates that the ethanolic extracts of Z. spina-christi and R. raetam possess significant antibacterial activity, with effects strongly dependent on the concentration. The superior performance of Z. spina-christi is consistent with reports attributing its activity to its high phenolic and flavonoid content (Kharma & Ismail, 2024).

Klebsiella pneumoniae exhibited the lowest susceptibility, consistent with its known resilience and multidrug-resistant phenotype (Edziri et al., 2021). As shown in Table 1, increasing the extract concentration led to a significant decrease in bacterial colony counts; for example, at 100% concentration, the mean count for S. aureus dropped to 12.28, whereas at 25% concentration, it rose to 17.03. This dose-dependent inhibition pattern is consistent with previous reports (Aboul-Enein et al., 2020; Musa et al., 2025), which found that higher extract concentrations effectively suppressed the growth of antibiotic-resistant bacteria.

Table 2 shows the clear differences in susceptibility among the tested bacterial species. *Klebsiella pneumoniae* showed the lowest susceptibility to the plant extracts, reflected by the highest mean value (20.07 ± 3.31), indicating reduced inhibition. *Escherichia coli* exhibited an intermediate response (18.74 ± 3.16), whereas *Staphylococcus aureus* was the most susceptible, with the lowest mean value (15.60 ± 2.90). This pattern aligns with the findings of Edziri et al. (2021), who reported that *Klebsiella* species often demonstrate greater resistance to plant-derived antimicrobial agents, highlighting the persistent challenge of multidrug-resistant strains.

As shown in Table 3, the comparative analysis between the two plant extracts demonstrated that *Ziziphus spina-christi* exhibited significantly greater antibacterial activity than *Retama raetam*, as indicated by the lower mean value (16.85 vs. 19.42; $p = 0.008$), reflecting stronger inhibition. This observation is consistent with the findings of Kharma and Ismail (2024), who reported that *Z. spina-christi*, owing to its rich phenolic profile, possesses enhanced antibacterial potency. This trend is further illustrated in Figure 1, where all tested bacterial species showed reduced growth when treated with *Z. spina-christi* extract compared to *R. raetam*.

A similar pattern was observed in Table 4, where the extract concentration demonstrated a clear inverse relationship with bacterial growth. Lower concentrations produced diminished inhibition, as shown by the increase in mean values at 25% concentration (20.79) compared to 100% concentration (15.83) ($p < 0.001$). These results corroborate the concentration-dependent effects reported by Benkhouili et al. (2022) and Elsabrouty et al. (2024), who highlighted the importance of extract strength in determining the antimicrobial impact.

Furthermore, the ANOVA results presented in Table 4 confirm the statistically significant differences among the three bacterial species ($F = 11.096$, $p = 0.001$). Tukey's HSD post hoc test indicates that *Staphylococcus aureus* differs significantly from both *Klebsiella* and *Escherichia coli*, while no

significant difference is observed between *Klebsiella* and *E. coli* (p = 0.412). These species-specific variations, also reflected in Figure 2, are consistent with the findings of Soriano et al. (2022), who emphasized that the efficacy of plant extracts is influenced by intrinsic bacterial characteristics.

The evaluation of the extract concentration effects in Table 6 further reinforces these patterns, with ANOVA confirming significant variation across concentrations ($F = 12.358$, $p < 0.001$). Games–Howell post hoc comparisons support this conclusion, highlighting the importance of optimizing the dosage to achieve maximal antibacterial activity, as recommended by Wallace et al. (2020). Moreover, the interactive effects between extract type and concentration, illustrated in Table 7 and Figure 3, reveal that *Z. spina-christi* exerts markedly stronger inhibition at higher concentrations—particularly against *S. aureus*—while *R. raetam* demonstrates comparatively greater activity against *E. coli* at lower concentrations. These differences align with the observations of Hammouche-Mokranea et al. (2017), who attributed the variability in antibacterial performance to distinct phytochemical profiles.

Overall, the findings summarized in Table 8 support the recommendations made by Noman et al. (2023) and Kamel et al. (2024), highlighting the potential of local medicinal plants as sources of natural antimicrobial agents. Nevertheless, further toxicological and clinical evaluations, as highlighted by Wallace et al. (2020), Musa et al. (2025), and Edziri et al. (2021), remain essential to ensure the safety and practical applicability of these extracts.

Conclusion

The findings of this study demonstrate that ethanolic extracts of Ziziphus spina-christi and Retama raetam exhibit notable antibacterial activity in a concentration-dependent manner against Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae. Overall, Z. spina-christi showed superior inhibitory effects, particularly against S. aureus, highlighting its strong antibacterial potential. These results suggest that both plant species are promising natural sources of complementary antibacterial agents that may support efforts to mitigate antimicrobial resistance and reduce dependence on conventional antibiotics.

Recommendations

Based on the current findings, several research directions are recommended to further elucidate the antibacterial potential of these extracts.

1. Determine Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC):Quantitative microdilution assays are needed to establish precise antibacterial potency.
2. Conduct Phytochemical Profiling:Identification and quantification of key bioactive compounds, such as phenolics, flavonoids, and alkaloids, would help correlate chemical composition with biological activity.
3. Perform In Vivo or Food-Model Studies:Such studies are essential to validate the safety, stability, and effectiveness of the extracts under realistic application conditions.
4. Testing should be expanded to include additional clinical and multidrug-resistant isolates to strengthen the relevance and generalizability of the findings, especially in clinical and public health contexts.
5. Investigate Formulation and Stability Enhancements:Developing optimized extract formulations, including nanoparticle-based delivery systems, may improve their activity and shelf stability.

Collectively, these steps will help advance the practical application of Z. spina-christi and R. raetam extracts as potential natural antibacterial agents in the future.

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