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Antibacterial effect of hydroethanolic *Annona muricata* leaf extract against *Streptococcus sanguinis* ATCC 10556: an in vitro study

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ABSTRACT

Background: The increasing interest in plant-derived antimicrobial compounds has encouraged the evaluation of *Annona muricata* as a potential source of bioactive substances against oral microorganisms. *Streptococcus sanguinis* is an early colonizer of dental biofilm and may participate in the establishment of complex oral microbial communities. **Objective:** To evaluate the in vitro antibacterial activity of three concentrations of hydroethanolic *Annona muricata* leaf extract against *Streptococcus sanguinis* ATCC 10556. **Methods:** An experimental in vitro study was conducted using hydroethanolic leaf extract at concentrations of 50%, 75%, and 100%, with eight replicates per group. Chlorhexidine 0.12% and ethanol 70% were used as positive and negative controls, respectively. Antibacterial activity was evaluated by agar disk diffusion, and inhibition-zone diameters were measured in millimeters. Differences among groups were assessed using the Kruskal–Wallis test. **Results:** Mean inhibition-zone diameters were 6.63 ± 0.52 mm, 13.06 ± 0.30 mm, and 16.44 ± 0.37 mm for the 50%, 75%, and 100% extracts, respectively. Chlorhexidine produced the largest inhibition zone (21.55 ± 1.23 mm), whereas ethanol produced 6.00 ± 0.00 mm. Overall differences among groups were statistically significant ($p < 0.001$). **Conclusions:** Hydroethanolic *Annona muricata* leaf extract exhibited concentration-dependent in vitro antibacterial activity against *S. sanguinis* ATCC 10556, with the greatest activity observed at 100%, although chlorhexidine showed the highest inhibitory effect.

Keywords: *Annona muricata*; Antibacterial Agents; Plant Extracts; *Streptococcus sanguinis*; Chlorhexidine.

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Introduction

Dental caries remains one of the most prevalent oral diseases worldwide and continues to represent a substantial public health burden. A global analysis based on the Global Burden of Disease 2019 estimated more than 2 billion prevalent cases of untreated caries in permanent teeth, confirming that the disease remains highly frequent despite improvements in preventive and restorative strategies [1]. Dental caries develops within a complex microbial ecosystem in which early colonizers contribute to the establishment and maturation of dental biofilms, creating the ecological conditions for subsequent microbial interactions involved in health and disease [2].

Oral streptococci are among the primary colonizers of dental surfaces and play a central role in shaping the composition and succession of the oral biofilm. *Streptococcus sanguinis* is a commensal member of this group that readily adheres to oral surfaces and participates in early biofilm formation. Although it is commonly associated with oral microbial homeostasis and may antagonize cariogenic microorganisms such as *Streptococcus mutans*, its capacity to form biofilms and interact with other species makes it an important organism for studying antimicrobial activity within the oral environment [2,3].

Interest in plant-derived antimicrobial compounds has increased because medicinal plants contain structurally diverse secondary metabolites with potentially useful biological activities. *Annona muricata* L., commonly known as soursop, contains several classes of bioactive compounds, including alkaloids, phenolic compounds, flavonoids, tannins, terpenoids, and acetogenins. Experimental and review evidence has described antibacterial activity among the pharmacological properties of this species, although the magnitude of the effect varies according to the plant part, extraction solvent, bacterial species, and concentration evaluated [4,5].

Recent in vitro studies have supported the antibacterial potential of *A. muricata* extracts against different microorganisms. Ethanolic and aqueous extracts obtained from several parts of the plant have shown inhibitory activity against biofilm-forming bacteria, while studies using ethanolic extracts have reported concentration-dependent effects against Gram-positive microorganisms [6,7]. Nevertheless, the available peer-reviewed evidence has predominantly evaluated bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and

other streptococcal species, whereas direct evidence concerning hydroethanolic *A. muricata* leaf extracts against *S. sanguinis* remains limited [5–7].

Assessing this interaction is relevant because the antibacterial effect of plant extracts may differ substantially among bacterial species and according to extract concentration. Determining the susceptibility of a standardized *S. sanguinis* strain to different concentrations of *A. muricata* leaf extract could therefore provide experimental evidence for future studies on plant-derived compounds intended for oral applications. Accordingly, the objective of this study was to evaluate the in vitro antibacterial effect of hydroethanolic *Annona muricata* leaf extract at concentrations of 50%, 75%, and 100% against *Streptococcus sanguinis* ATCC 10556.

Materials and methods

Study design and setting

A quantitative, laboratory-based, experimental in vitro study was conducted to evaluate the antibacterial effect of hydroethanolic *Annona muricata* leaf extract at three concentrations against *Streptococcus sanguinis* ATCC 10556. The experimental phase was carried out in the Pharmacognosy and Microbiology laboratory facilities of the Universidad Nacional de Trujillo, Trujillo, Peru, during 2019. The study followed a completely randomized parallel-group design with post-intervention measurement of bacterial growth inhibition. Because this was a laboratory in vitro experiment involving a reference bacterial strain and plant material, reporting guidelines intended for observational studies, clinical trials, systematic reviews, case reports, diagnostic-accuracy studies, and qualitative research were not applicable.

Experimental units and sample size

The experimental units consisted of Petri dishes inoculated with *S. sanguinis* ATCC 10556. Five experimental conditions were evaluated: hydroethanolic *A. muricata* leaf extract at 50%, 75%, and 100%; 0.12% chlorhexidine as the positive control; and 70% ethanol as the negative control.

Sample size was determined for comparison of quantitative outcomes using an α level of 0.05 ($Z_{\alpha/2} = 1.96$), a β error of 0.20 ($Z_{\beta} = 0.84$), and an assumed standard deviation equivalent to 0.7 times the expected difference between group means. The calculation yielded eight replicates per experimental condition.

Consequently, 40 experimental units were analyzed, with eight replicates in each of the five groups.

Eligibility criteria

Petri dishes adequately inoculated with *S. sanguinis* ATCC 10556 and showing appropriate bacterial growth under the established laboratory conditions were included. Plates showing contamination or alterations attributable to contamination during the experimental procedure were excluded.

Study variables

The independent variable was the concentration of hydroethanolic *A. muricata* leaf extract, evaluated at 50%, 75%, and 100%. The dependent variable was antibacterial activity against *S. sanguinis* ATCC 10556, operationalized as the diameter of the growth-inhibition zone expressed in millimeters. Chlorhexidine 0.12% and ethanol 70% were included as positive and negative controls, respectively.

Plant material and preparation of the hydroethanolic extract

Approximately 1 kg of *A. muricata* leaves was collected in Trujillo, La Libertad, Peru. A complete botanical specimen was submitted to the Herbarium Truxillense of the Universidad Nacional de Trujillo for taxonomic identification. Leaves in adequate condition, without visible fungal damage, discoloration, or wilting, were selected.

The plant material was washed with abundant water and disinfected using sodium hypochlorite at 80 ppm. The leaves were spread on kraft paper and dried in a forced-convection oven at 40 °C. After drying, the material was ground and passed through a No. 20 mesh sieve and subsequently stored in wide-mouth amber glass containers.

For extraction, 100 g of powdered leaf material was placed in an amber glass container and covered with an ethanol–water mixture (70:30, v/v) approximately 2 cm above the plant material. The mixture was macerated for 7 days and manually agitated for 15 minutes twice daily. After maceration, the preparation was vacuum-filtered through Whatman No. 1 filter paper. The resulting hydroethanolic filtrate was concentrated using a rotary evaporator and subsequently dried in an oven at 40 °C until a dry extract was obtained. From this extract, concentrations of 50%, 75%, and 100% were prepared.

Preparations were stored in amber glass containers under refrigeration at 4–8 °C until microbiological testing.

Bacterial strain and inoculum standardization

A lyophilized reference strain of *S. sanguinis* ATCC 10556 was used. The strain was reactivated in 50 mL of Brain Heart Infusion broth and incubated at 37 °C for 24–48 hours under microaerophilic conditions. Culture purity was verified by streaking onto blood agar, followed by incubation under the same conditions and Gram staining of a morphologically compatible colony. A pure colony was subsequently transferred to Brain Heart Infusion broth and tryptic soy agar for maintenance before antibacterial testing.

For inoculum preparation, the strain was cultured on tryptic soy agar at 37 °C for 24 hours under reduced-oxygen conditions. Three to four isolated colonies were suspended in Brain Heart Infusion broth or sterile physiological saline until turbidity equivalent to a 0.5 McFarland standard was achieved, corresponding to approximately 1.5×10^8 colony-forming units/mL. The standardized bacterial suspension was used within 15 minutes of preparation.

Antibacterial susceptibility assay

Antibacterial activity was evaluated using an agar disk-diffusion procedure. Diffusion-based assays enable the comparative assessment of antimicrobial activity by quantifying growth-inhibition zones around antimicrobial-containing disks [8].

A 100-μL aliquot of the standardized *S. sanguinis* suspension was distributed uniformly onto Mueller–Hinton agar plates. Sterile swabs were passed over the agar surface in three directions to obtain homogeneous bacterial inoculation, and plates were allowed to stand at room temperature for 3–5 minutes to absorb excess surface moisture.

Sterile Whatman No. 3 filter-paper disks were impregnated with 50 μL of hydroethanolic *A. muricata* extract at concentrations of 50%, 75%, or 100% and placed onto the inoculated agar surface using sterile forceps. Chlorhexidine 0.12% was used as the positive control and ethanol 70% as the negative control. Plates were incubated in an inverted position at 37 °C under reduced-oxygen conditions using a GasPak jar and candle method. Antibacterial activity was determined from the diameter of the inhibition zone surrounding each disk.

Measurement of antibacterial activity

The primary outcome was the diameter of the bacterial growth-inhibition zone, expressed in millimeters. Measurements were obtained using a calibrated digital Vernier caliper (Mitutoyo, model 500-157-30) and recorded in a data collection form specifically designed for the study. Because the outcome consisted of a direct physical measurement rather than a psychometric construct, content-validity or internal-consistency testing was not applicable. Calibration of the measuring instrument constituted the relevant quality-control procedure.

Statistical Analysis

The eight raw measurements available for each of the five experimental conditions were included in the analysis. Inhibition-zone diameters were summarized using descriptive measures of central tendency and dispersion. Because the experimental groups were small and the study records documented departure from normality, overall differences among experimental conditions were assessed using the Kruskal–Wallis test. When the global test was statistically significant, pairwise comparisons were performed using the Mann–Whitney U test with Holm adjustment for multiple comparisons. Statistical significance was established at $p < 0.05$.

Ethical considerations

The study involved only a standardized bacterial reference strain and plant material and did not include human participants, identifiable human information, patient-derived biological samples, or experimental animals. Consequently, informed consent and the ethical requirements specifically applicable to research involving human participants were not applicable. Institutional authorization was obtained to conduct the experimental procedures in the corresponding laboratory facilities. The study was conducted in accordance with the institutional principles of scientific integrity and responsible research practice in force at the time of the investigation.

Results

A total of 40 experimental units were analyzed, with eight replicates for each of the three concentrations of hydroethanolic *Annona muricata* leaf extract and for

each control condition. No experimental units were reported as lost or excluded from the final analysis.

The largest mean inhibition zone was observed with 0.12% chlorhexidine (21.55 ± 1.23 mm), followed by the 100% *A. muricata* extract (16.44 ± 0.37 mm), the 75% extract (13.06 ± 0.30 mm), and the 50% extract (6.63 ± 0.52 mm). The 70% ethanol control produced a mean inhibition zone of 6.00 ± 0.00 mm. Median inhibition-zone diameters followed the same numerical order (Table 1).

Overall inhibition-zone diameters differed significantly among the five experimental conditions (Kruskal–Wallis $H = 37.18$, $df = 4$, $p < 0.001$). In Holm-adjusted pairwise comparisons, the 100% extract differed significantly from the 75% and 50% extracts and from both control conditions. The 75% extract also differed significantly from the 50% extract, chlorhexidine, and ethanol. A statistically significant difference was additionally observed between the 50% extract and the ethanol control (Table 2).

Discussion

The main finding of this study was that hydroethanolic *Annona muricata* leaf extract exhibited antibacterial activity against *Streptococcus sanguinis* ATCC 10556, with progressively larger inhibition zones as extract concentration increased. The 100% extract showed the greatest activity among the three tested concentrations, whereas 0.12% chlorhexidine produced the largest inhibition zone overall. These findings support a concentration-related antibacterial response under the experimental conditions evaluated; however, because this was an in vitro study based on agar diffusion, the results should not be interpreted as evidence of clinical efficacy.

The antibacterial activity observed is consistent with previous investigations of *A. muricata*. Rodrigues Batista et al. reported inhibitory activity of methanolic extracts against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, with marked variation according to the plant fraction and dilution used [9]. The concentration-dependent tendency observed in the present study is also compatible with the findings already reported by Castro Hernández et al. [7], who found increasing inhibition of *S. aureus* and β -hemolytic streptococci as the concentration of ethanolic *A. muricata* leaf extract increased. Thus, although the bacterial species and extraction procedures were

Table 1. Inhibition-zone diameters of hydroethanolic *Annona muricata* leaf extract against *Streptococcus sanguinis* ATCC 10556.

Experimental condition	n	Mean $\pm$ SD, mm	Median (IQR), mm
A. muricata 50%	8	6.63 $\pm$ 0.52	7.00 (6.00–7.00)
A. muricata 75%	8	13.06 $\pm$ 0.30	12.90 (12.88–13.25)
A. muricata 100%	8	16.44 $\pm$ 0.37	16.50 (16.15–16.73)
Chlorhexidine 0.12%	8	21.55 $\pm$ 1.23	22.05 (21.48–22.25)
Ethanol 70%	8	6.00 $\pm$ 0.00	6.00 (6.00–6.00)

Note: SD, standard deviation; IQR, interquartile range. Overall comparison: Kruskal–Wallis $H = 37.18$, $df = 4$, $p < 0.001$.

Table 2. Pairwise comparison of inhibition-zone diameters among experimental conditions

Comparison	Mann–Whitney U	Adjusted p value*
A. muricata 100% vs 75%	64.0	0.005
A. muricata 100% vs 50%	64.0	0.005
A. muricata 100% vs chlorhexidine 0.12%	0.0	0.005
A. muricata 100% vs ethanol 70%	64.0	0.004
A. muricata 75% vs 50%	64.0	0.005
A. muricata 75% vs chlorhexidine 0.12%	0.0	0.005
A. muricata 75% vs ethanol 70%	64.0	0.004
A. muricata 50% vs chlorhexidine 0.12%	0.0	0.005
A. muricata 50% vs ethanol 70%	52.0	0.011
Chlorhexidine 0.12% vs ethanol 70%	64.0	0.004

Note: p values were adjusted for multiple pairwise comparisons using the Holm procedure. Statistical significance was defined as adjusted $p < 0.05$.

different, both studies support the premise that antibacterial activity is influenced by the amount and composition of extract applied.

Differences among studies are likely related in part to extraction methodology. Cagnini et al. evaluated an *A. muricata* leaf oleoresin obtained by supercritical carbon dioxide extraction and demonstrated bactericidal activity against several bacterial species, with minimum inhibitory concentrations ranging from 0.0025 to 0.010 mg/mL [10]. In contrast, the present study used hydroethanolic maceration with a 70:30 ethanol–water mixture. The extraction solvent influences the spectrum and concentration of recovered phytochemicals because compounds differ in polarity and solubility. Therefore, inhibition-zone diameters obtained with hydroethanolic preparations cannot be directly compared with those obtained using methanolic, ethanolic, aqueous, ethyl-acetate, or supercritical-fluid extracts [5,10].

Variability in antimicrobial response is also evident across bacterial species. Ngemenya et al. found that aqueous and ethanolic preparations derived from different parts of *A. muricata* produced heterogeneous

antibacterial effects against biofilm-forming methicillin-resistant *S. aureus*, and some extracts modified the activity of conventional antibiotics [11]. This reinforces that antibacterial activity cannot be attributed solely to the botanical species; plant part, extraction procedure, test microorganism, concentration, and experimental method all influence the measured response [5,6,11].

The biological activity of *A. muricata* preparations has also been demonstrated beyond bacterial planktonic models. Campos et al. showed that an ethanolic leaf extract had antifungal activity against multidrug-resistant *Candida albicans* and affected structures associated with the fungal cell envelope. Although these findings cannot be extrapolated directly to *S. sanguinis*, they demonstrate that the antimicrobial activity of the plant is not restricted to a single microbial group and that the biological response depends on the chemical profile of the extract and the target microorganism [12].

The same research group subsequently demonstrated antibiofilm activity of ethanolic *A. muricata* extract against multidrug-resistant *C. albicans* [13]. This aspect is relevant to oral research because microorganisms

embedded in biofilms can respond differently from planktonic cells. The present experiment assessed growth inhibition using an agar-diffusion model and therefore cannot establish whether the extract would retain the same activity against mature *S. sanguinis* biofilms or multispecies oral communities. Future testing under biofilm conditions is consequently necessary before extrapolating these findings to the oral environment.

The effect of *A. muricata* may also vary substantially according to the microorganism being exposed. Meza-Gutiérrez et al. reported that *A. muricata* leaf extracts could promote the growth of *Lactobacillus casei* under certain experimental conditions rather than inhibit it [14]. This apparently contrasting behavior is important because it demonstrates that plant extracts may exert species-specific effects rather than acting as indiscriminate antimicrobial agents. Therefore, the inhibition of *S. sanguinis* observed in the present study should be understood as a response of this particular standardized strain under the specific concentrations and conditions evaluated.

This consideration is particularly important because *S. sanguinis* is not merely a pathogenic target. It is a pioneer colonizer of dental surfaces and a common component of the healthy oral biofilm. Hassan et al. confirmed the biofilm-forming capacity of *S. sanguinis* and identified genes potentially involved in this phenotype [15]. Moreover, contemporary understanding of oral commensal streptococci indicates that these microorganisms contribute to microbial succession, competition, and ecological stability in the oral cavity [2,3]. Consequently, demonstrating antibacterial activity against *S. sanguinis* does not by itself establish that suppressing this organism would be clinically advantageous.

The comparison with chlorhexidine should also be interpreted within this ecological context. In the present experiment, 0.12% chlorhexidine produced the greatest inhibition zone and was more active than all three concentrations of *A. muricata*. Chlorhexidine is a potent broad-spectrum oral antiseptic; however, evidence reviewed by Brookes et al. indicates that its use may alter the composition and diversity of the oral microbiome and modify salivary biochemical parameters [16]. Thus, the fact that the plant extract showed less inhibition than chlorhexidine does not demonstrate therapeutic equivalence, but neither should antibacterial performance be judged exclusively by maximal suppression of a commensal microorganism.

Another relevant finding was the comparatively small inhibition zone produced by the 50% extract. Although it remained statistically different from the 70% ethanol control after adjustment for multiple comparisons, the absolute difference was modest. This emphasizes the importance of including a solvent control in plant-extract studies, because ethanol itself possesses antimicrobial activity and may contribute to the measured inhibition. The use of both positive and negative controls therefore strengthened interpretation of the present experiment and allowed the effect of the extract to be distinguished more clearly from that of its vehicle [8].

Recent experimental work with *A. muricata* leaves further supports the importance of concentration and extraction conditions. Wilkie et al. evaluated an ethyl-acetate leaf extract against several bacterial strains and observed antibacterial activity that varied according to extract concentration and microorganism [17]. These results are consistent with the overall pattern found here but also illustrate why quantitative comparison of inhibition zones across studies must be made cautiously when solvents, bacterial strains, concentrations, inoculum conditions, and susceptibility assays differ.

The phytochemical complexity of *A. muricata* provides a plausible basis for these variations. Recent reviews describe alkaloids, annonaceous acetogenins, flavonoids, phenolic compounds, terpenes, and other metabolites among the constituents of the species [4,18]. These classes of compounds may differ substantially in abundance according to geographical origin, plant tissue, maturity, extraction solvent, and processing. Consequently, the concentration percentages evaluated in this study represent crude extract concentrations rather than standardized concentrations of individual antibacterial molecules.

Research has also explored the use of *A. muricata* constituents in antimicrobial nanomaterials. Vindhya et al. synthesized cobalt-doped zinc oxide nanoparticles using *A. muricata* leaf extract and reported antimicrobial activity together with antioxidant and cytotoxicity assessments [19]. Although nanoparticle-based preparations are fundamentally different from the crude hydroethanolic extract evaluated here, these studies illustrate the broader interest in *A. muricata*-derived compounds as components of antimicrobial systems. They also reinforce that formulation can substantially modify biological activity and therefore must be evaluated independently before any dental application is proposed.

Standardization is another key issue for future translational research. Chan et al. evaluated commercially available *A. muricata* leaf products and identified substantial variation in annonacin concentration, acetogenin profiles, labeled doses, and quality indicators [20]. Such variability demonstrates that botanical material cannot be assumed to have uniform chemical composition. In the context of the present findings, future studies should therefore incorporate phytochemical characterization and quantification of relevant bioactive constituents rather than relying exclusively on percentage concentrations of crude extract.

From a scientific and dental perspective, the present study provides preliminary evidence that hydroethanolic *A. muricata* leaf extract can inhibit *S. sanguinis* ATCC 10556 under controlled laboratory conditions. Nevertheless, its potential application should be framed cautiously. Before considering incorporation into mouthrinses, gels, dentifrices, intracanal formulations, or other dental products, it would be necessary to determine minimum inhibitory and bactericidal concentrations, establish chemical composition and stability, evaluate cytotoxicity and biocompatibility, and investigate activity against clinically relevant oral biofilms. Particular attention should also be paid to microbial selectivity because preserving beneficial oral commensals may be as important as suppressing disease-associated microorganisms [2,3,16].

Among the strengths of this study were the use of a standardized ATCC reference strain, clearly defined concentrations, a positive antimicrobial control, a solvent control, eight replicates per experimental condition, standardized inoculum preparation, and direct measurement of inhibition-zone diameters using a calibrated digital instrument. In addition, the final statistical approach used a non-parametric overall comparison followed by multiplicity-adjusted pairwise testing, which was more appropriate for the distribution of the experimental data than the originally proposed parametric post hoc procedure.

Several limitations should be considered. First, only one reference strain was tested, which restricts extrapolation to clinical isolates and other oral microorganisms. Second, antibacterial activity was evaluated only by agar diffusion; minimum inhibitory concentration, minimum bactericidal concentration, and time-kill kinetics were not determined. Third, the hydroethanolic extract was not chemically characterized, preventing identification or quantification of the compounds responsible for the

observed activity. Fourth, cytotoxicity and biocompatibility with oral tissues were not evaluated. Fifth, the agar-based assay did not reproduce the structural and ecological complexity of mature mono- or multispecies oral biofilms. Finally, only three relatively high extract concentrations were tested, limiting precise characterization of the concentration–response relationship.

Future studies should therefore chemically characterize the hydroethanolic extract, quantify its main bioactive constituents, evaluate a broader concentration range, and determine minimum inhibitory and bactericidal concentrations. Experiments using clinical isolates and mono- and multispecies oral biofilms are also needed. Cytotoxicity testing in relevant oral cell lines should precede translational development, while comparative experiments should determine whether *A. muricata*-derived compounds preferentially inhibit disease-associated organisms without adversely affecting commensal species. These approaches would clarify whether the antibacterial activity demonstrated in the present study can be developed into a biologically selective and clinically relevant oral-health application.

Conclusions

Hydroethanolic *Annona muricata* leaf extract exhibited *in vitro* antibacterial activity against *Streptococcus sanguinis* ATCC 10556, with greater inhibition observed as the extract concentration increased. The 100% concentration showed the highest antibacterial activity among the tested extracts, whereas 0.12% chlorhexidine produced the greatest overall inhibition. These findings demonstrate that *A. muricata* leaf extract has measurable antibacterial activity under the experimental conditions evaluated, although its inhibitory effect remained lower than that of chlorhexidine.

Author Contributions Statement (CRediT)

API: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft, Visualization, and Project Administration, Revision and Editing.

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Conflict of Interest

The author declares no financial, institutional, professional, or personal conflicts of interest that could have influenced the conduct or publication of this study.

Data Availability

The data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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