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Inhibitory effect of dietary phenolic compounds on *Chlamydia pneumoniae* in cell cultures

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EB, elementary body
HL cells, human cell line
IFU, inclusion-forming unit
LDH, lactate dehydrogenase
RB, reticulate body
CHD, coronary heart disease
MIC, minimum inhibitory concentration

ABSTRACT

Chlamydial infections are very common worldwide. All chlamydial species have a tendency to cause persistent infections, which have been associated to several chronic diseases including blinding trachoma, infertility and coronary heart disease (CHD). At present, no efficient treatment for the eradication of chronic chlamydial infections exists and, thus, new antichlamydial compounds are urgently needed. This study was designed to screen antichlamydial activity of natural flavonoids and other natural and structurally similar synthetic compounds against *Chlamydia pneumoniae* in human cell line (HL).

HL cells were infected with *C. pneumoniae* and incubated 72 h with studied compounds. Reduction in the number of inclusions was determined with immunofluorescence staining. In vitro minimum inhibitory concentration was also determined for some of the most active compounds. Thirty seven percentage of the studied compounds (57 in total) were highly active against *C. pneumoniae* and all the studied compounds were non-toxic to the host cells at studied concentrations.

Our study revealed direct antichlamydial effect for selected polyphenolic compounds against *C. pneumoniae*, in vitro. We also demonstrated the ability of some of the investigated compounds to accumulate inside cells or into cell membranes and cause inhibition, even when present only prior to infection.

1. Introduction

Chlamydia are obligate intracellular gram-negative bacteria with a unique developmental life cycle, including an infective metabolically inactive elementary body (EB) and a metabolically active reticulate body (RB), which multiplies inside the inclusion in the host cell cytoplasm. Chlamydial species have a tendency to cause persistent infections, which gradually, sometimes over a course of tens of years, may lead to severe sequelae.

Chlamydia pneumoniae is an established important respiratory pathogen [1], and it seems to be a very common infectious

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agent: seroepidemiological studies worldwide indicate that virtually everyone becomes infected at least once during their lifetime. This bacterium is a common cause of acute upper and lower respiratory tract infections, including pharyngitis, sinusitis and pneumonia [2]. It also has a tendency to cause chronic infections, and there is augmenting evidence of the involvement of chronic *C. pneumoniae* infection in atherosclerotic diseases, including coronary heart disease (CHD) [3–6]. *C. pneumoniae* has also been associated with asthma, chronic obstructive pulmonary disease and lung cancer [7,8]. Even though acute *C. pneumoniae* infections can be successfully treated with several antibiotics, eradication of chronic *C. pneumoniae* infection seems extremely difficult. High doses and prolonged treatment are often needed to achieve clinical cure, and there is still a risk of the persistence of *C. pneumoniae* in the tissues after treatment. In fact, *C. pneumoniae* can be recovered from cell cultures even after 30-day antibiotic treatment [9], and, in animal models, infection persists after monotherapy [10,11]. Also, recent, large-scale randomized placebo-controlled antibiotic trials have shown that even prolonged antibiotic treatments do not affect *C. pneumoniae* antibody titers (see reviews [12,13]). Thus, it is extremely important to find new compounds that can be used in the treatment or prophylaxis of *C. pneumoniae* infections.

Only plants and micro-organisms are capable of biological synthesis of the aromatic nucleus, which is the basic structure of plant phenolic compounds, such as flavonoids, produced via the shikimic acid pathway [14]. Flavonoids are polyphenolic substances with a flavan nucleus consisting of 15 carbon atoms arranged in three rings (C6–C3–C6), which generally occur as glycosylated derivatives. A large number of antimicrobial substances, called phytoalexins (including flavonoids), are found in nature, and they make up a variable group of compounds playing important roles in the natural defence of several organisms. Flavonoids can be found in, for example, fruits, vegetables and plant-derived beverages, such as tea and wine. The bioavailability of different polyphenols can vary greatly. The plasma concentrations of flavonoids with the best bioavailability profiles are about 5 μmol/l, whereas the corresponding values for polyphenols with poor bioavailability profiles (monomeric flavonols, flavones and flavanols) are usually less than 1 μmol/l [15]. However, it is possible that some phenolic compounds accumulate inside specific target tissues causing higher local concentration compared to their plasma concentrations [16]. Because the elimination of polyphenols from the body is generally quite fast, they must be consumed daily in order to maintain high concentrations. The effects of flavonols and flavones on enzymes regulating cell division and proliferation, platelet aggregation, detoxification as well as inflammatory and immune responses are well documented [17,18]. Phenolic compounds can interfere with the initiation and promotion of tumour growth [19], and a resent study has also revealed some of the molecular mechanisms responsible for their cancer prevention properties [20]. Number of naturally occurring, as well as synthetic flavonoids, have also potent anti-HIV activity in vitro [21]. Quercetin, a widely studied flavonoid, is reported to prevent lung cancer [22]. Due to their phytoestrogenic properties, flavonoids, as well as other plant phenolic compounds, are implicated to prevent menopausal symptoms, osteoporosis, breast and ovarian cancer as well as heart disease [23,24], and epidemiological studies have actually demonstrated a beneficial role of flavonoids regarding to the risk of CHD [25,26]. Recently, the polyphenols in tea have been shown to inactivate *C. pneumoniae* in vitro [27], and the antichlamydial activity of at least one flavonoid (luteolin) has also been demonstrated in vivo [28]. In the present study, we evaluated the effects of several groups of natural phenolic compounds and synthetic compounds derived from natural ones on *C. pneumoniae* infection in cell cultures. The promising results of this study generated a patent concerning natural-, synthetic compounds and plant extracts in the treatment and/or prevention of a chlamydial infection [29]. In past, rather poor bioavailability of phenolic compounds has complicated their antichlamydial efficacy studies in humans, since it is not easy to obtain adequate amounts of these compounds from normal diet. This could, and hopefully will, be overcome by using patented medicinal preparations, food additive compositions or functional foodstuffs containing high concentrations of these compounds and monitoring their antichlamydial efficacy and gained health effects in humans.

2. Materials and methods

2.1. Tested compounds and sample preparation

The compounds used in this study are presented in Fig. 1, and they were purchased from: Acros (USA), Avocado (England), Carl Roth GmbH (Germany), Extrasynthese (France), Fluka (Switzerland), ICC Chemical Corporation (USA), Merck (Germany), and MP Biomedicals (USA) or Sigma (Germany). Pure compounds (Fig. 1) were dissolved in dimethylsulphoxide (DMSO) to concentrations of 25 mM (stock solution) on the day of inoculation. For the preparation of test solutions, 5 μl of the stock solution was added to 2.5 ml of the culture medium used in the incubation of infected cells, yielding a 50 μM final concentration (based on preliminary test results).

2.2. Cell line

HL cells [30], which are conventionally used in *C. pneumoniae* cultivation, were grown in RPMI 1640 medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% foetal bovine serum (FBS, South American Origin, BioWhittaker Europe, Verviers, Belgium), 2 mM glutamine and 20 μg streptomycin ml^{-1} (HL medium).

2.3. Chlamydial strain

C. pneumoniae, isolate Kajaani 7 (K7), was propagated in HL cells, and chlamydial elementary bodies were purified from the cells by Urografin (Schering Ag, Berlin, Germany) gradient ultracentrifugation. Purified elementary bodies were suspended in sucrose-phosphate–glutamic acid [0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4, and 5 mM L-glutamic acid (pH 7.4)] buffer and stored in small aliquots at −70 °C until used. The number of chlamydial inclusion-forming units (IFUs) was determined by infecting confluent HL cell layers growing on coverslips in 24-well plates with 10-fold serial

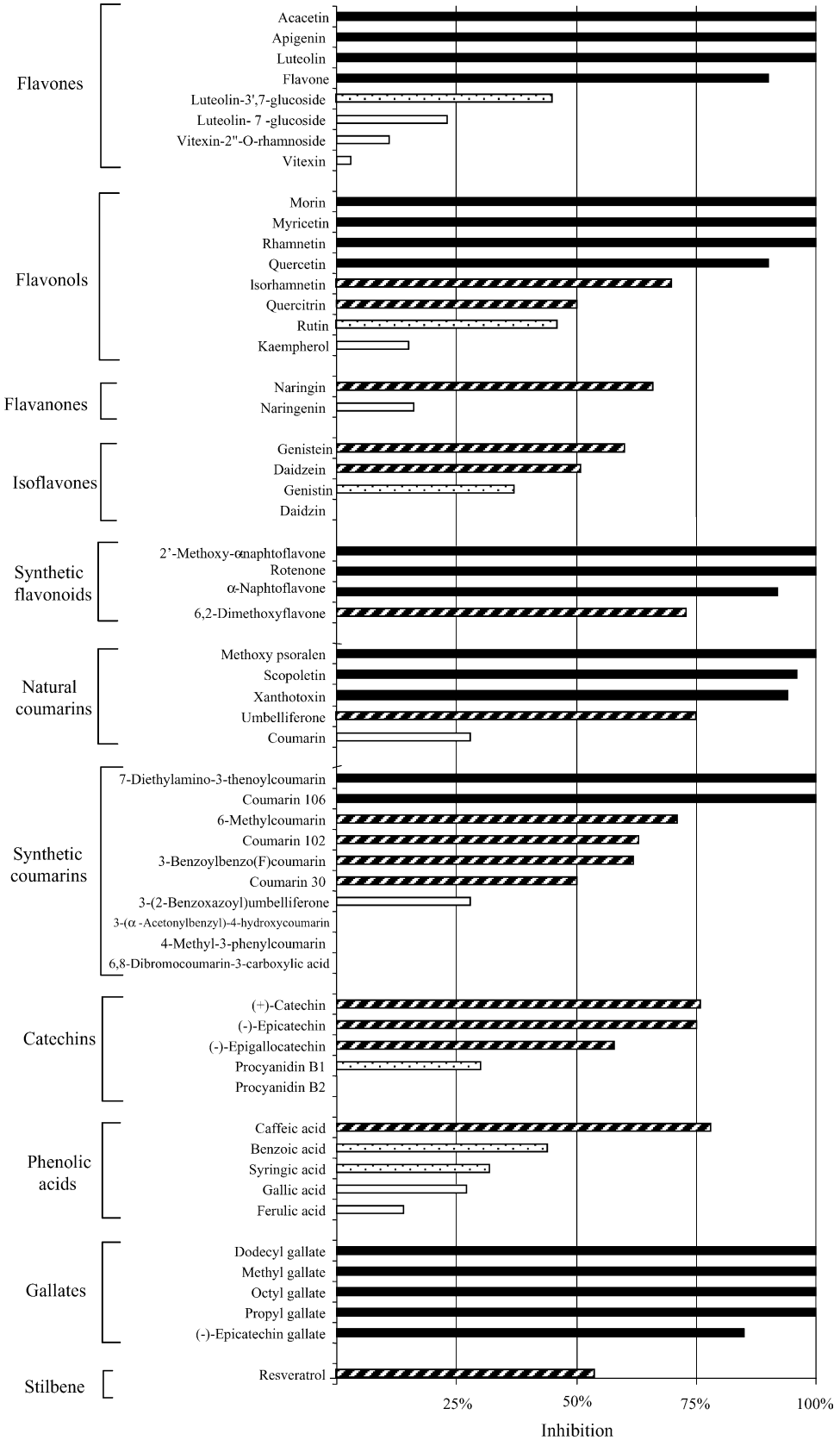


Fig. 1 – Average inhibition percentages of natural or natural-based compounds and some synthetic derivatives against *C. pneumoniae* at 50 μM concentration (n = 4 or more). The categories of activity are determined as: highly active (black bar) = 85–100% inhibition compared to DMSO-controls; active (striped bar) = 50–84%; moderately active (black dotted bar) = 30–49%; inactive (white bar) = <30%.

dilutions of the stock solution and by counting inclusions after 72 h incubation and immunofluorescence staining as described below. The *C. pneumoniae* EB preparations and the cell line were negative for mycoplasma DNA as determined using a commercial polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Inoculation of cells

HL cells (450,000 cells/well) were allowed to attach onto the coverslips in 24-well plates, and the cell monolayer cultures were infected 24 h later. K7 isolate was added to the infection medium (HL medium containing 0.5 μg cycloheximide ml^{-1}; an 80-s ribosome inhibitor) in a concentration of 10^3 IFU/well. The plates were centrifuged at 20 °C, 550 × *g*, 60 min (Heraeus Multifuge 3 S, Kendro Laboratory Products GmbH, Hanau, Germany). The medium was aspirated, and the infected cells were cultured in fresh infection medium containing a 50 μM final concentration of the test compound at 37 °C, 95% humidity and 5% CO_2 for 72 h.

2.5. Immunofluorescence staining and counting of chlamydial inclusions (inhibition)

Seventy Two hours after the infection, the medium was removed from the wells, and the coverslips were washed once with PBS and fixed in methanol for 10 min. The coverslips were allowed to dry, and the chlamydial inclusions were stained directly with fluorescein isothiocyanate (FITC)-conjugated chlamydial genus-specific antibody (PathFinder® *Chlamydia* Culture Confirmation System, Bio-Rad S.A., WA, USA). The stained inclusions were examined under a fluorescence microscope (Nikon ECLIPSE TE300 inverted microscope with TE-FM epi-fluorescence attachment, Tokyo, Japan). In each sample, the number of inclusions was counted from four random microscopic fields (400× magnification). Inhibition percentage was determined on the basis of the average number of inclusion bodies per eight microscopic fields from four parallel samples and calculated by comparing the number of inclusions in a treated sample to the number of inclusions in a DMSO control using the following formula:

$$\left[\frac{\text{inclusions in control} - \text{inclusions in treated sample}}{\text{inclusions in control}}\right] \times 100$$

2.6. Infective yield

To analyse the infectivity of *C. pneumoniae* EBs after compound treatment, infected HL cells were cultivated directly on the bottom of 24-well plates in the presence of the test compound or without any compound (positive control). The cells were mechanically disrupted after 72 h incubation and repassaged on freshly prepared confluent HL cell layers (on coverslips) and centrifuged at 550 × *g* for 60 min. The infected cells were cultured in the infection medium (without test compound) at 37 °C, 95% humidity and 5% CO_2 for 72 h, after which the inclusions were stained and counted as described above and compared to the number of inclusions in positive control.

2.7. Pre-treatment of HL cells prior to infection

In order to analyse whether compounds can induce inhibition in *C. pneumoniae* growth when present only prior to infection, HL cells were pre-incubated for 24 h with the investigated compound before actual *C. pneumoniae* infection. The infection was done according to the infection protocol, and the infected cells were grown in the infection medium without the test compound for 72 h before the inclusions were stained, counted and compared to the DMSO control as described above.

2.8. Minimum inhibitory concentration (MIC)

In vitro, minimum inhibitory concentration was determined by increasing the compound concentrations progressively, starting from 5 μM, up to the concentration where no inclusions could be found. Ofloxacin, an antibiotic known to be effective against *C. pneumoniae*, was used as a positive control.

2.9. Cytotoxicity of compounds

The cytotoxicity of selected compounds to HL cells was examined using a commercial lactate dehydrogenase (LDH) test kit (CytoTox®, Promega Corp., USA). HL cells were grown overnight in 96-well plates. The medium was aspirated and the cells were washed twice with HBSS wash solution. One hundred microlitres of the 25 mM compound solution used in the antichlamydial activity studies were added to 1 ml of HBSS for a short 2 h exposure, or 1 ml of HL media was added for a 72 h exposure ($n = 8$ wells). LDH levels were determined after 2 or 72 h and compared to the negative control.

Dead cells were visually counted after 72 h co-incubation with the tested substance. The medium was aspirated from the 24-well plates, and 200 μl of sucrose-phosphate–glutamine medium (SPG) were added into the wells. The cells were scraped from the bottom of each well, and the suspension was collected into a test tube. Ten microlitres of 0.4% Trypan blue (Trypan blue 0.4%, Sigma Chemical Co., UK) was added into a test tube containing 30 μl of the cell suspension. The solution was mixed, and both viable and dead cells were counted using a Buerker chamber (FORTUNA®, Germany, Tiefe depth 0.100 mM and 0.0025 mm^2).

3. Results

Both natural phenolic compounds and synthetic compounds derived from natural ones showed high activity against *C. pneumoniae*: 37% (21/57) of the compounds were highly active; 28% (16/57) active; 11% (6/57) moderately active; 24% (14/57) inactive. Highly active compounds were found in many compound groups, but the most active group was that of gallates. Inactive compounds could also be found in many compound classes, but among synthetic coumarins, many compounds had 0% inhibition. The activity of all tested compounds is presented in Fig. 1, and the MICs for the most effective compounds are shown in Table 1. All compounds in this study were non-toxic to the host cells, according to the

Table 1 – In vitro MICs of some of the most active natural or natural-based compounds for *C. pneumoniae*

Compound	MIC (μM)
Acacetin	29
Dodecyl gallate	18
Luteolin	8.8
Methoxypsoralen	50
Methyl gallate	29
Morin	50
Myricetin	29
Octyl gallate	29
Propyl gallate	50
Rhamnetin	50
Ofloxacin (control)	1.4

CytoTox® kit (viability of HL cells was over 95% for all tested compounds) as well as the Trypan blue test (results not shown).

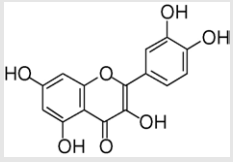
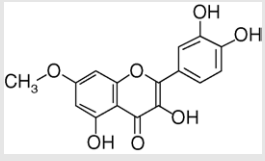
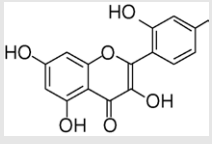
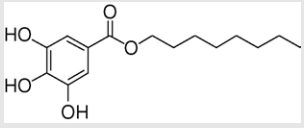
To analyse the infectivity of EBs after treatment in vitro, infective yield was determined for four active compounds (Table 2; infective yield), and their capability to retain or penetrate membrane was examined with *C. pneumoniae* and HL cells (Table 2; pre-treatment). Pre-treatment of HL cells with all of the four phenolic compounds tested decreased the infectivity of *C. pneumoniae* to 0–50% of that seen in untreated controls (Table 2). When the compounds were continuously present in cell cultures, infectivity was clearly lower, varying from 0 to 32%. All compounds also decreased the infective yields, and the most chlamydiosidic compound was rhamnetin, which killed *C. pneumoniae* at both concentrations tested.

Table 2 – The effect of pre-treatment and continuous treatment on formation of chlamydial inclusions and yield of infectious chlamydial particles

Compound (μM)	*C. pneumoniae*		
	Pre-treatment	Continuous treatment	Infective yield
Quercetin			
50	10 (90)	10 (90)	24 (76)
0.5	23 (77)	32 (68)	42 (58)
Rhamnetin			
50	41 (59)	0 (100)	0 (100)
0.5	50 (50)	27 (73)	0 (100)
Morin			
50	4 (96)	0 (100)	6 (94)
0.5	38 (62)	20 (80)	19 (81)
Octyl gallate			
50	0 (100)	0 (100)	0 (100)
0.5	38 (62)	18 (82)	41 (59)

Values are average percentages of remaining inclusions compared to untreated controls 72 h post-infection. Inhibition percentages in parenthesis.

Table 3 – Literature based, experimental or calculated, physico-chemical properties of quercetin, rhamnetin, morin and octyl gallate

Compound	Structure	log *P*	Water solubility (mg/l)	MW
Quercetin [34]		1.48	60.0	302.2
Rhamnetin [34]		2.04	ND	316.3
Morin [35]		1.54	250.0	338.3
Octyl gallate [36]		3.6	36.0	282.3

ND, not determined.

4. Discussion

Since antichlamydial activity was seen in various compound groups, detailed structural analysis was performed in order to elicit some features common for both active and inactive molecules. Flavones and flavonols have the same basic structure, and both groups contain both active and inactive compounds. Among flavones and flavonols, structure is related to activity in such a way that all the compounds with 50% or less inhibition contain a sugar moiety or moieties as substituents, whereas none of the more active compounds (over 70% inhibition) contain sugars. This basic structure of flavones and flavonols without any sugars seems to be highly active against *C. pneumoniae* compared to the similar basic structure of flavanones or isoflavones. The same phenomenon can also be seen among synthetic flavonoids, a highly active group of compounds, where three out of four compounds are derived from flavone. One very common group of compounds in nature is coumarins, which were shown in this study to be highly active against *C. pneumoniae*. Coumarin itself is inactive, but substituents in the benzene ring enhance activity, as shown for example by methoxypsoralen, which blocks *C. pneumoniae* growth completely.

Most antibiotics are designated to kill metabolically active bacteria—RBs in the case of *Chlamydia*—by inhibiting protein or nucleic acid synthesis, and they hence have no effect on the metabolically inactive chlamydial EBs. However, total elimination of *Chlamydia* is important to prevent the development of chronic infection. In the present study, we could show that several phenolic compounds, and especially rhamnetin, are highly chlamydiosidic against *C. pneumoniae*, even when present at low concentrations.

The flavonols quercetin, rhamnetin and morin have the same basic three-ring structure (Table 3), which has been shown to be capable of penetrating phospholipid membranes [31], but they also have small structural differences, which may influence the mechanisms by which they cause inhibition. This is best seen in the production of infective EBs, which can be totally blocked by rhamnetin, while after treatment with quercetin and morin, inclusions can be found after repassage (Table 2; infective yield). This difference is probably attributable to the methoxy group in the A-ring of rhamnetin, which makes this molecule more hydrophobic, since it is the only structural difference between rhamnetin and quercetin (Table 3).

Octyl gallate differs structurally and has different physicochemical properties compared to the other compounds shown in Table 3. For this reason, it most likely also causes inhibition by a different mechanism. Octyl gallate has been shown to inhibit nuclear factor kappaB (NF-kappaB) [32], an ubiquitous transcription factor known to be active during *C. pneumoniae* infection [33]. When the cell is infected, this factor is translocated into the nucleus, where it activates several genes encoding cytokines and is necessary for an effective host defence response. Therefore, when NF-kappaB and, subsequently, the host defence response are attenuated, it would be expected to enhance infection, but, in our study, a totally opposite result was seen: octyl gallate is highly active against *C. pneumoniae*.

In conclusion, we used the *C. pneumoniae* K7 strain to demonstrate the antichlamydial activity of several phenolic compounds, and four of the most active compounds were further evaluated in pre-treatment and infective yield tests for more information about their inhibition mechanism. The structural comparison between active and inactive compounds elicited no specific structure that could make these compounds active, but it did reveal some activity-enhancing structures, such as lack of sugars, as substituents of flavones or flavonols basic structure. Our study clearly showed that structurally different phenolic compounds have antichlamydial activity against *C. pneumoniae* in human cells. We also demonstrated that some of these compounds have the ability to accumulate inside cells or into cell membranes and cause inhibition, even when present only prior to infection. Thus, it is possible that, in the future, some of these phenolic compounds could be used in the treatment and prophylaxis of *C. pneumoniae* infections.

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