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Attenuation of Oxidative Stress and Anti-Alzheimer Effect of Ursolic Acid

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline and neurodegeneration, with oxidative stress playing a pivotal role in its pathophysiology. Ursolic acid (UA), a triterpenoid found in various medicinal plants, exhibits antioxidant and neuroprotective properties that may counteract oxidative damage associated with AD. This study aimed to evaluate the antioxidant, neuroprotective, and anti-Alzheimer effects of Ursolic acid using in vitro assays. The antioxidant potential was assessed via the DPPH free radical scavenging assay. The neuroprotective effects were evaluated through acetylcholinesterase inhibition assays, while UA's anti-Alzheimer potential was examined using amyloid-beta aggregation and beta-secretase inhibition assays. Ursolic acid demonstrated significant ($p<0.001$) antioxidant activity, effectively scavenging DPPH radicals in a concentration-dependent manner. In the acetylcholinesterase inhibition assay, UA exhibited a notable ($p<0.05$) reduction in enzyme activity from 10 μM to the maximum concentration of 80μM, suggesting its potential to enhance cholinergic neurotransmission. Furthermore, UA significantly inhibited amyloid-beta aggregation and reduced beta-secretase activity between concentrations of 10 μM – 80 μM, indicating its promising role in mitigating key pathological features of Alzheimer's disease. The findings suggest that Ursolic acid possesses potent antioxidant and neuroprotective effects, along with the ability to inhibit amyloid-beta aggregation and beta-secretase activity. These results highlight the therapeutic potential of Ursolic acid as a candidate for the prevention and treatment of Alzheimer's disease, warranting further investigation in vivo models to validate its efficacy and mechanisms of action.

Keywords: *Alzheimer's Disease, Antioxidant, Neuroprotection, Oxidative Stress, Ursolic Acid.*

Introduction

Alzheimer's disease is the most common neurodegenerative disorder worldwide, affecting millions of people and accounting for the majority of dementia cases. It is characterized by progressive memory loss, cognitive decline, and behavioural alterations that significantly impair daily functioning and quality of life [1]. The disease is pathologically characterized by the accumulation of amyloid-beta plaques and neurofibrillary tangles which consist of hyperphosphorylated tau proteins, in the brain. These hallmark features of AD lead to neuronal loss, synaptic dysfunction, and eventually, widespread neurodegeneration. Additional mechanisms, including oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, and cholinergic

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deficits, have been implicated in the pathogenesis of AD [2, 3]. The pathophysiology of Alzheimer's disease is multi-faceted, with amyloid-beta plaque deposition and tau protein aggregation being central to disease progression. Aβ plaques trigger neurotoxicity by promoting oxidative stress and activating inflammatory pathways, while tau tangles disrupt neuronal communication, contributing to synaptic loss and apoptosis. Additionally, mitochondrial dysfunction and the overproduction of reactive oxygen species lead to cellular damage and further neuronal death [4, 5].

Current pharmacological treatments for AD primarily include acetylcholinesterase inhibitors (e.g., donepezil, rivastigmine, galantamine) and NMDA receptor antagonists, which target symptoms but do not halt disease progression. These drugs aim to improve cognitive function or slow disease progression temporarily but are limited by their inability to address the underlying causes of AD [6]. The multifactorial nature of AD makes it challenging to treat, and despite significant advancements in understanding the disease, no cure currently exists. In recent years, there has been growing interest in natural compounds with neuroprotective properties for treating neurodegenerative diseases [7]. Ursolic acid, a natural compound found in various plants such as apples, rosemary, and holy basil, has been studied for its wide range of therapeutic effects, particularly in reducing oxidative stress [8]. Ursolic acid has already been reported to have a range of pharmacological activities, including anti-inflammatory, antioxidant, and neuroprotective effects, making it a promising compound for further exploration in the context of neurodegenerative diseases [9].

Ursolic acid (UA), a pentacyclic triterpenoid, is one such compound that has gained attention due to its diverse pharmacological properties. UA is widely found in various plants, including apples (*Malus domestica*), rosemary (*Rosmarinus officinalis*), and holy basil (*Ocimum sanctum*) [10]. It belongs to the triterpene class of phytochemicals, which are known for their bioactive properties. Ursolic acid has been traditionally used in herbal medicine and is known for its anti-inflammatory, antioxidant, anti-cancer, and anti-microbial effects [11]. These properties make UA an attractive candidate for investigating its potential in preventing or mitigating neurodegenerative conditions, particularly Alzheimer's disease, where oxidative stress and inflammation are key drivers of disease progression [12].

Despite the promising pharmacological properties of ursolic acid, its anti-Alzheimer potential remains relatively underexplored, particularly concerning its ability to reduce oxidative stress and inhibit the progression of amyloid-beta pathology. Most existing studies focus on its general neuroprotective effects or its benefits in conditions unrelated to AD. There is a significant research gap in understanding the mechanisms by which ursolic acid can attenuate oxidative stress in Alzheimer's pathology, as well as its role in reducing amyloid plaque formation and mitigating neuroinflammation. Our study aims to investigate the neuroprotective potential of ursolic acid in the context of Alzheimer's disease, focusing specifically on its ability to reduce oxidative stress, inhibit amyloid-beta aggregation, and improve neuronal survival.

Materials and Methods

In-vitro Lipid Peroxidation (LPO) Inhibition Assay

The free radical scavenging activity of Ursolic acid was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [13]. A 0.004% DPPH solution was prepared in methanol, and 10 µL of Ursolic acid, at varying concentrations (1, 2.5, 5, 10, 20, 40 & 80 µM), was added to 190 µL of the DPPH solution. The mixture was vortexed and incubated at 37°C for 20 minutes. A control blank, containing only the solvent without the test compound or

standard, was prepared for comparison. The reduction in absorbance, resulting from the scavenging of DPPH free radicals by the test compound, was measured at 517 nm. The IC_{50} value, representing the concentration at which 50% of the DPPH radicals were quenched, was determined. Each experiment was performed in triplicate, and the percentage inhibition was calculated accordingly. Ascorbic acid was used as the reference standard for comparison.

In-vitro Acetylcholinesterase (AChE) Inhibition Assay

Ursolic acid and the standard Donepezil hydrochloride were prepared at different concentrations (1, 2.5, 5, 10, 20, 40, 80 µM) using 0.05 M tris base to examine their AChE inhibitory activities [14]. Briefly, 200 µl of acetylthiocholine iodide (15 mM), 1000 µl of DTNB (3 mM), and 200 µl of Ursolic acid or standard Donepezil hydrochloride at various concentrations were mixed and incubated for 15 minutes at 30°C. The mixture was then monitored spectrophotometrically at 412 nm, 10 times at 13-second intervals. Following this, 200 µl of AChE solution (0.3 U/ml) was added to the initial mixture to start the reaction, and the absorbance was determined. The control contained all components except the test compounds and standard drugs.

Assessment of Aβ (1–42) Concentration: Preparation of Aβ Solution

The Aβ solution was prepared following a previously published method [15]. Synthetic β-Amyloid Peptide 1-42 (Aβ1-42) (PP69, Sigma Merck, USA) was dissolved in 0.1% ammonia solution to a final concentration of 250 µM and was sonicated in ice-cold water for a total of 5 minutes (1 minute × 5 times) to prevent pre-aggregation. Aliquots of Aβ were then diluted to 25 µM in 50 mM phosphate buffer (pH 7.5) and 100 mM NaCl for the preparation of the Aβ working solution.

Thioflavin T Fluorescence Assay

The thioflavin T (ThT) fluorescence assay was performed [16]. Aβ solution (8 µL) was mixed with different concentrations of ursolic acid and the standard Donepezil hydrochloride. The mixture was then added to 1.6 mL of ThT solution containing 5 µM ThT and 50 mM NaOH-glycine buffer (pH 8.5). The samples were incubated at 37°C, and the fibrillogenesis rate was monitored using the ThT fluorescence assay. The ThT fluorescence levels of the samples were evaluated using a Biotek Synergy H4 hybrid multimode reader (USA). The respective excitation and emission wavelengths were set at 446 nm and 490 nm.

In-vitro Inhibition Study on β-Secretase (BACE1) Enzyme

The β-Secretase enzyme inhibitory assessment was conducted using the BACE1 fluorescence assay [17]. In brief, 10 µL of BACE1 enzyme solution at a concentration of 1.0 unit/mL (Thermo Fisher Scientific, USA), 10 µL of the test compound Ursolic acid and the standard Donepezil hydrochloride, and 10 µL of 750 nM β-secretase substrate IV (Calbiochem, Merck, Darmstadt, Germany) were mixed in the reaction wells. The reaction mixture was incubated for 1 hour at ambient temperature. Fluorescence readings were then recorded at 380 nm (excitation) and 510 nm (emission) using the Biotek Synergy Hybrid H4 multimode reader (Molecular Devices, USA).

Statistical Analysis

Data were analyzed using GraphPad Prism (version 7.0). The results were expressed as Mean ± SEM, and the IC50 values were obtained from the linear regression plots. Two-way ANOVA was used to assess differences between means at a significance level of $p < 0.001$. The means were compared with the standard groups using the Holm-Sidak test.

Results

Effect of Ursolic Acid on DPPH Free Radical Formation

The DPPH assay results (Figure 1) demonstrated a concentration-dependent increase in the percentage inhibition of DPPH radicals by Ursolic acid. The activity was compared to Ascorbic acid, used as the reference standard. At the highest concentration (80 µM), Ursolic acid exhibited an inhibition of approximately 90%, comparable to the inhibition observed with Ascorbic acid. Lower concentrations of Ursolic acid (1 µM and 2.5 µM) showed minimal inhibition (less than 20%), while a gradual increase in activity was noted at concentrations above 5 µM, reaching significant inhibition around 40 µM. These results suggest that Ursolic acid is an effective scavenger of DPPH radicals in a dose-dependent manner, with similar potency to Ascorbic acid.

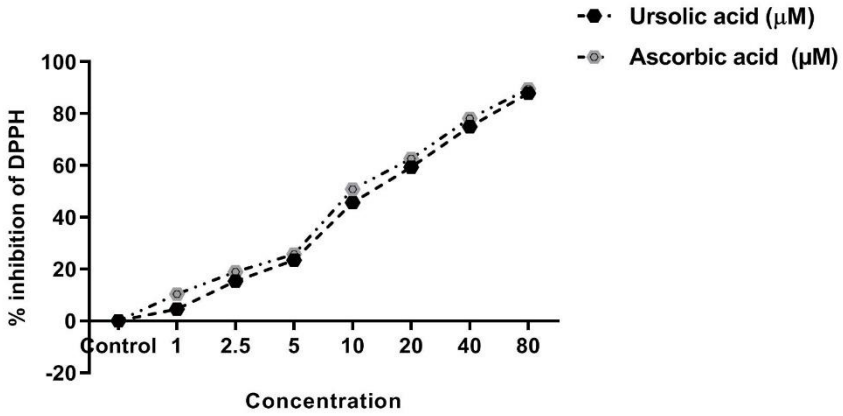

Figure 1. Effect of Ursolic Acid on DPPH Free Radical Scavenging Activity

Effect of Ursolic Acid on Acetylcholinesterase Activity

The inhibitory effect of Ursolic acid on acetylcholinesterase (AChE) activity was analyzed and compared to Donepezil, a standard AChE inhibitor (Figure 2). Ursolic acid exhibited a concentration-dependent increase in AChE inhibition, with inhibition levels reaching over 80% at 80µM. Similarly, Donepezil showed potent inhibition, with both compounds demonstrating almost parallel inhibition curves. At lower concentrations (1µM and 2.5µM), the inhibition was moderate but steadily increased at higher concentrations. The data suggest that Ursolic acid has significant potential as an AChE inhibitor, comparable to Donepezil.

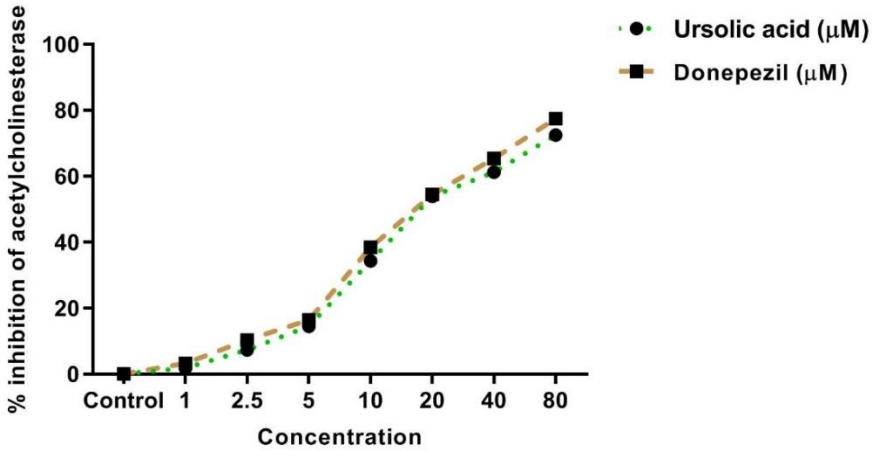

Figure 2. Effect of Ursolic Acid on Acetylcholinesterase Activity

Effect of Ursolic Acid on β-secretase Activity

The inhibition of β-secretase (BACE-1) by Ursolic acid was compared with Donepezil (Figure 3). A concentration-dependent inhibition was observed, with both Ursolic acid and Donepezil showing substantial inhibition of BACE-1 at 80µM, nearing 100%. At lower concentrations (1 µM to 10 µM), both compounds demonstrated moderate inhibitory activity, but significant inhibition was observed beyond 20 µM. The results indicate that Ursolic acid is a potent inhibitor of BACE-1, with similar efficacy to Donepezil at higher concentrations.

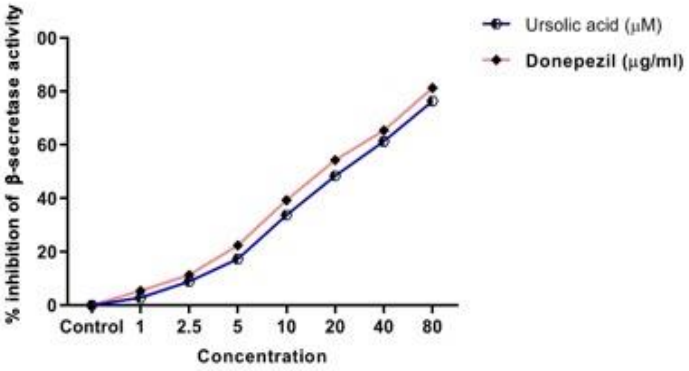

Figure 3. Effect of Ursolic Acid on β-secretase Activity

Effect of Ursolic Acid on *In-vitro* Aβ42 Aggregation

The graph (Figure 4 & Figure 5) presents the effects of different concentrations of Ursolic acid and Donepezil on the aggregation of amyloid-beta (Aβ 1-42), a peptide linked to Alzheimer's disease. The % aggregation of Aβ is plotted against the concentrations (µM) of Ursolic acid and Donepezil. Both Ursolic acid and Donepezil demonstrate a dose-dependent inhibition of Aβ aggregation. At lower concentrations (1 µM to 5 µM), there is minimal reduction in Aβ aggregation, with both compounds showing similar effectiveness around 100% aggregation. As the concentration increases (from 10 µM to 80 µM), both compounds exhibit a significant reduction in Aβ aggregation. At 80 µM, the aggregation level drops to approximately 20-30% for both Ursolic acid and Donepezil, indicating that they are similarly effective at this higher concentration. The overall trend suggests that increasing concentrations of either compound result in a similar inhibition of Aβ aggregation, with a comparable reduction pattern for both.

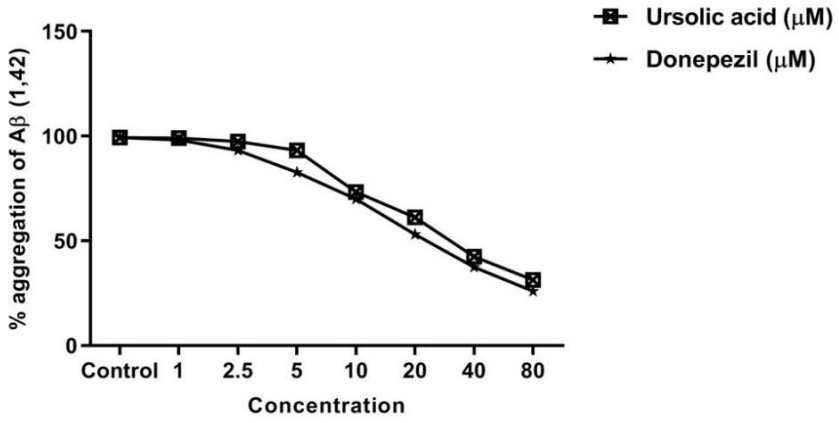

Figure 4. Effect of Ursolic Acid on $A\beta_{42}$ Aggregation

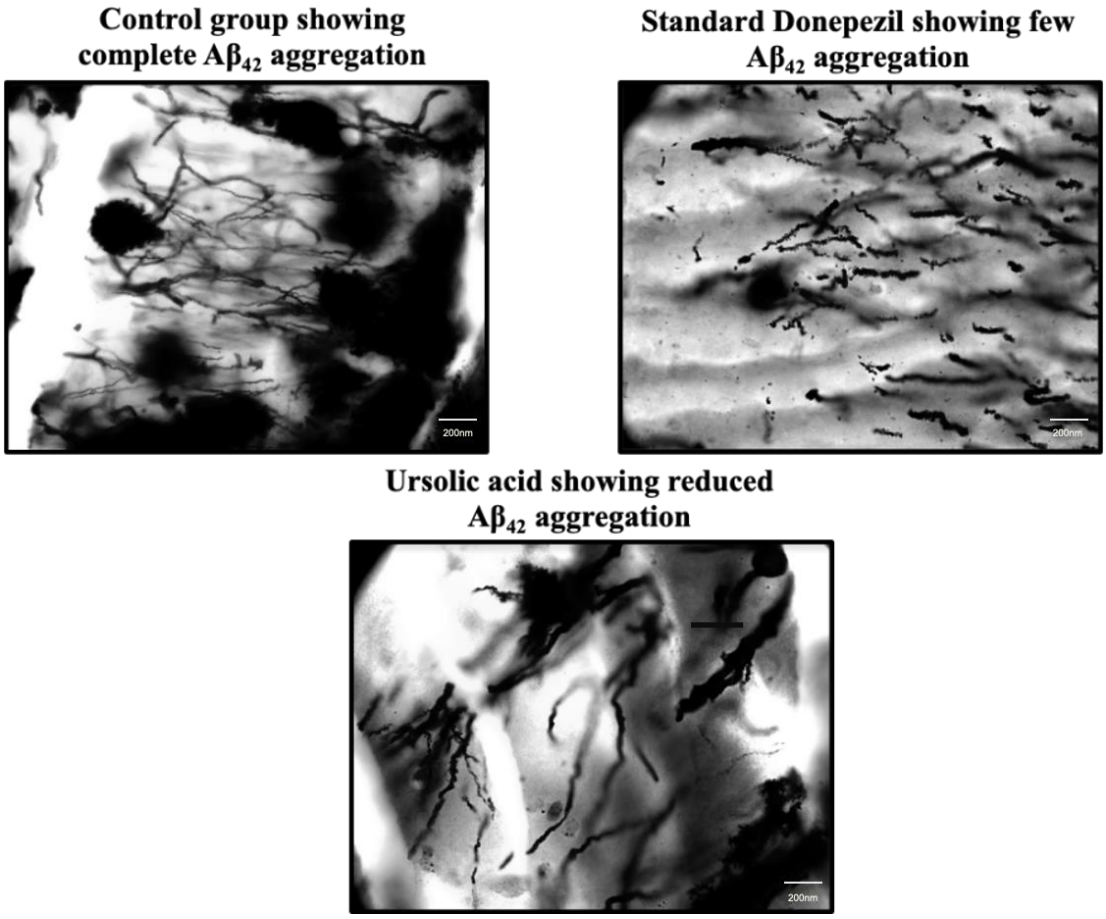

Figure 5. Representative TEM Images Showing the Effect of Ursolic Acid on $A\beta_{42}$ Aggregation

Discussion

Ursolic acid (UA), a natural triterpenoid, has demonstrated significant potential in multiple biological pathways, especially for its antioxidant, neuroprotective, and anti-Alzheimer's properties. This study evaluated these properties through well-established *in vitro* assays.

The antioxidant effect of UA was assessed using the DPPH free radical scavenging assay, a reliable method for evaluating the radical scavenging potential of compounds. Ursolic acid exhibited notable free radical scavenging activity, suggesting its ability to neutralize reactive oxygen species (ROS). Excessive ROS generation has been closely linked to cellular oxidative stress, a key contributor to neurodegeneration, ageing, and Alzheimer's disease (AD) pathogenesis. The antioxidant effect of UA could be attributed to its structural ability to donate hydrogen atoms to stabilize free radicals, as also reported by several studies on triterpenoids [18, 19]. Moreover, it has been demonstrated that UA reduces oxidative stress markers such as lipid peroxidation and protein carbonylation in neuronal models, reinforcing its role as an antioxidant [20].

In addition to its antioxidant activity, UA demonstrated neuroprotective potential by inhibiting acetylcholinesterase (AChE), an enzyme responsible for breaking down acetylcholine. Acetylcholine is a neurotransmitter crucial for cognitive function, and its reduction is a hallmark of Alzheimer’s disease. By inhibiting AChE, UA may help preserve acetylcholine levels, thereby potentially improving memory and cognitive functions. The current study's findings align with earlier reports that identified UA as a potent AChE inhibitor [21-23]. Furthermore, the dual action of UA in both antioxidant and acetylcholinesterase inhibition pathways

presents a comprehensive mechanism for its neuroprotective effects, supporting its therapeutic potential for AD [23].

The anti-Alzheimer's effects of UA were further confirmed through its inhibitory activity against amyloid-beta (Aβ) aggregation and beta-secretase activity. Aβ plaques are one of the primary pathological markers of AD, and their accumulation disrupts synaptic function, leading to neurodegeneration. UA significantly inhibited Aβ aggregation, suggesting that it may prevent or reduce the formation of these toxic plaques. This result is consistent with studies showing that compounds with antioxidant properties, like UA, often exhibit anti-amyloidogenic effects by interacting with Aβ peptides and inhibiting their aggregation [24, 25]. The ability of UA to modulate amyloid genesis positions it as a promising candidate in anti-Alzheimer therapy.

The inhibition of beta-secretase (BACE1), a key enzyme involved in the formation of Aβ peptides, provides further support for the anti-Alzheimer's potential of UA. BACE1 cleaves the amyloid precursor protein (APP) to produce Aβ peptides and inhibition of this enzyme has been a targeted strategy in AD drug development. The observed beta-secretase inhibition by UA suggests its role in attenuating the pathological cascade leading to Aβ plaque formation [26]. This dual mechanism—interfering with both Aβ aggregation and beta-secretase activity—makes UA a compelling candidate for addressing multiple facets of Alzheimer's pathology [20].

Overall, the results from this study align with previous research, reinforcing the therapeutic potential of Ursolic acid for neurodegenerative diseases, especially Alzheimer's. The synergistic effects of its antioxidant, acetylcholinesterase inhibitory, and anti-amyloidogenic properties provide a holistic approach toward mitigating oxidative stress and AD progression. Future research should focus on in vivo studies to better understand the pharmacokinetics, bioavailability, and long-term efficacy of UA in neurodegenerative models, as well as its potential to synergize with existing therapies.

Conclusion

In conclusion, this study highlights the promising therapeutic potential of Ursolic acid (UA) as an antioxidant, neuroprotective, and anti-Alzheimer agent through various in vitro assays. The findings demonstrate UA's ability to scavenge free radicals, inhibit acetylcholinesterase, and reduce amyloid-beta aggregation while also interfering with beta-secretase activity. These mechanisms collectively support UA's role in mitigating oxidative stress and addressing key pathological features associated with Alzheimer's disease. Given the multifaceted effects of UA, it stands out as a potential candidate for further investigation in the context of neurodegenerative disorders. Future studies, including *in vivo* experiments and clinical trials, are warranted to validate these findings and explore the therapeutic applications of Uric acid in Alzheimer's disease management. Overall, this research contributes to the growing body of evidence supporting the use of natural compounds in combating neurodegeneration and enhancing cognitive health.

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