http://dergipark.org.tr/tr/pub/anatolianbryology

DOI: 10.26672/anatolianbryology.1434173

Anatolian Bryology
Anadolu Briyoloji
Dergisi
Research Article
e-ISSN:2458-8474
Online

Determination of Biochemical Content and Antioxidant Activity of *Calliergonella cuspidata* (Hedw.) Loeske

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Received: 09 February 2024 **Revised: 04 March 2023** **Accepted: 08 March 2024**

Abstract

Since ancient times, humans have been utilizing various plants for medicinal purposes, a practice that has persisted from early civilizations to the present day. Plants serve as rich sources of biomolecules, although many of their contents remain unidentified. Bryophytes are considered important reservoirs for new natural products, with mosses being less explored compared to liverworts despite their broader species diversity. This study contains the content analysis of ethanol, methanol, and n-hexane extracts of *Calliergonella cuspidata* to predict and compare their biochemical compound profiles. The ethanol extract revealed the presence of 3-Formyl-N-methyl-9-[phenylethynyl]dibenzo2,3-a:5,6-a'-thiazine and Eicosane, while Beta-Elemene and Neophytadiene were identified in the methanol extract, and predominantly alkanes were found in the n-hexane extract. Subsequently, antioxidant activity was determined using the DPPH method with the ethanol extract, yielding an EC_{50} value of 1.0237 mg/ml.

Keywords: Bryophytes, Moss, *Calliergonella cuspidata*, GC/MS, Antioxidant, Türkiye

Calliergonella cuspidata (Hedw.) Loeske'nin Biyokimyasal İçeriğinin ve Antioksidan Aktivitesinin Belirlenmesi

Öz

İnsanların çok eski çağlardan beri çeşitli amaçlarla yararlandığı bitkilerin tıbbi amaçlı kullanımı ilk uygarlıklardan günümüze kadar devam etmiştir. Bitkiler oldukça zengin biyomolekül kaynakları oluştururlar. Ancak birçoğunun içerik tanımları henüz yapılmamıştır. Briyofitlerin yeni doğal ürünler için önemli bir rezervuar olduğu düşünülmektedir. Karayosunları ise daha geniş bir tür çeşitliliğine sahip olmasına rağmen, ciğerotlarına göre daha az araştırılmıştır. Bu çalışma, biyokimyasal bileşik profillerini tahmin etmek ve karşılaştırmak için *Calliergonella cuspidata*'nın etanol, metanol ve n-heksan ekstraktlarının içerik analizini içermektedir. Etanol ekstraktında 3-Formil-N-metil-9-[feniletinil]dibenzo[2,3-a:5,6-a'](1,4)-tiazin ve Eikosan, metanol ekstraktında Beta-Elemen ve Neofitadien, N-hekzan ekstraktında ise ağırlıklı olarak alkanlara rastlanmıştır. Daha sonra etanol ekstraktı ile DPPH yöntemi kullanılarak antioksidan aktivite belirlenmiştir. EC_{50} değeri 1,0237 mg/ml olarak tespit edilmiştir.

Anahtar kelimeler: Briyofitler, Karayosunları, *Calliergonella cuspidata*, GC/MS, Antioksidan, Türkiye

1. Introduction

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To cite this article: *Turu D. Bozkurt S.D. Yaman C. Gül G. Benek A. Canli K. 2024. Determination of Biochemical Content and Antioxidant Activity of Calliergonella cuspidata (Hedw.) Loeske. Anatolian Bryology. 10:1, 25-33.*

In nature, all living organisms coexist in a delicate balance. Plants, too, have interacted with humans since the beginning of humanity (Gezgin, 2006). The medicinal use of plants, which humans have utilized for various purposes since ancient times, has persisted from the earliest civilizations to the present (Emre, 2012). The content of these plants, which offer numerous benefits, has always been a subject of curiosity. Through years of research, the constituents of plants have been elucidated, and primary and secondary metabolites produced by plants have become fundamental products in industry. The primary reason for the widespread preference for these products lies in the plant's ability to convert minerals and certain elements into compounds that the human body can absorb in its metabolism. Additionally, these natural products derived from plants positively impact the functions of specific tissues and organs in the organism (Faydaoğlu and Sürücüoğlu, 2011; Tilkat et al., 2021).

Medicinal plants have drawn much attention because of their antioxidant qualities and possible contributions to the prevention of chronic illnesses like cancer, heart disease, and neurological problems. The pathophysiology of these disorders has been linked to oxidative stress, which is caused by an imbalance between the body's antioxidant defenses and the formation of reactive oxygen species (ROS). Antioxidants originating from plants, such as terpenoids, phenolic acids, and flavonoids, can neutralize ROS and shield cells from oxidative damage. This has sparked great interest in exploring the antioxidant capabilities of medicinal plants for their potential to prevent a range of chronic diseases (Pandey and Rizvi, 2009; Lobo et al., 2010).

Scientists are actively researching ways to add new antioxidants to existing ones and obtain more effective antioxidants by employing various methods and materials. Mosses are recognized as one of the significant materials showing promise for future use in antioxidant activity research. In a previous study, it has been found that 1 mg of moss extract exhibits a reducing power equivalent to a commonly used standard antioxidant (Bhattarai et al., 2008). The antioxidant potential of the moss used in the study is believed to originate from the active compounds it possesses.

Plants constitute highly rich sources of biomolecules. However, while the contents of some of these have been disclosed, many plant ingredients still contain unidentified substances. Therefore, ensuring accurate and comprehensive characterization of bioactive compounds present in medicinal plants and determining the quantities of essential components for treatment is crucial (Martins et al., 2015; Lorini et. al., 2021; Alawode et al., 2021).

Bryophytes constitute the second-largest diversity among green terrestrial plants (Asakawa, 2007). Centuries ago, the Chinese and Native Americans found that moss could effectively heal wounds and lower infection rates. Mosses have been the subject of in-depth research and application in a wide range of fields since their discovery (Benek et al., 2022). In light of these consequences, bryophytes are believed to represent a significant source of novel natural compounds. Hundreds of novel phytochemicals have been isolated and identified from bryophytes (Bandyopadhyay and Dey, 2022; Commisso et al., 2021). Despite mosses having a broader species diversity, they have been less studied than liverworts due to their lower oil content. While it is known that only a few moss species contain substantial amounts of terpenoids, bibenzyls, flavonoids, fatty acids, and acetophenones, the content of some remains still undetermined (Asakawa et al., 2013).

This study involves the content analysis of ethanol, methanol, and n-hexane extracts of *Calliergonella cuspidata* to predict and compare the biochemical compound profiles. Gas Chromatography-Mass Spectrometry (GC-MS), one of the most advanced and robust technologies, was employed for this purpose. Subsequently, the antioxidant activity was determined using the DPPH method with the *C. cuspidata* ethanol extract.

2. Materials and Methods

2.1. Chemicals

1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich, ascorbic acid from Carlo Erba, and ethanol, methanol, and n-hexane from Merck.

2.2. Collection localities

C. cuspidata was collected from Akdağ, Amasya, Türkiye (N 40° 46.322′ E 035° 54,672′) and identified by Prof. Dr. Kerem CANLI. The moss sample was brought to the laboratory by placing it in a sample bag. After drying the sample at room temperature with air, it was stored (Herbarium no: CANLI007) until experiments were conducted at the Fauna and Flora Research and Application Center (FAMER), Dokuz Eylül University, Buca, Izmir, Türkiye.

2.3. Preparation of extracts from *Calliergonella cuspidata*

For the extraction of active compounds from the ground moss sample, 5 grams were transferred into 200 ml of ethanol, methanol, and n-hexane, respectively. Active compounds were extracted by shaking at room temperature and 160 rpm for three days. Following this, filtered extracts evaporated at 35-40°C under a vacuum using a rotary evaporator, yielding 0.035 g, 0.030 g and 0.038 g dried mass, respectively (Altuner et al., 2014).

To be used for antioxidant activity, the ethanol extract prepared at a concentration of 1 mg/ml. An equal concentration of ascorbic acid was also prepared to be used as a positive control. Lastly, the extracts prepared for GC-MS analysis were passed through 0.45 μm injector filters to remove any residual particles before analysis.

2.4 Biochemical screening

Biochemical analyses were carried out according to Canli et al. (2023). In this study, GC-MS analysis was conducted using Agilent GC 8890-Agilent GC/MSD 5977B (Agilent Technologies Inc., USA). Helium was used as carrier gas and component identification was achieved by matching retention times with Wiley-Nist MS data libraries. Chemical components in quantities greater than 0.5% were considered major components. GC/MS analyses were repeated for accuracy, and some parameters were modified based on the solvents used.

2.5 Determination of antioxidant activity

The free radical scavenging activity of *C. cuspidata* ethanol extract was tested in terms of its ability to bleach the stable DPPH. This assay relies on the conversion of the deep violet color of the DPPH solution, measured at 515 nm, to yellow due to the neutralization of stable free DPPH radicals by antioxidant molecules (Kedare and Singh, 2011). 0.0039 g DPPH was mixed with ethanol. A 96-well plate containing DPPH solution and the extract at concentrations ranging from 7.8125 to 1000 μg/mL was kept in the dark at room temperature for 30 minutes. After 30 minutes, the plate's absorbance was measured at λ = 515 nm with using a micro-plate reader (Biotek Microplate Spectrophotometer, USA). As a positive control, the commercially known antioxidant ascorbic acid was used. All experiments were conducted in triplicate.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation (SD) of three independent experiments for each antioxidant; following the statistical interpretation of data, EC_{50} values were expressed as 95% confidence interval with Four Parameter Logistic Regression (Chen et al., 2013). The results analyzed using One-Way ANOVA (Analysis of Variance) and Pearson correlation tests in the R Studio (version 2023.12.1). The significance level was set at $p \leq 0.05$.

3. Results

3.1 Biochemicals in extracts

The identified substances from GC/MS analysis are presented in Table 1.

Table.1 Biochemical screening of *C. cuspidata*

Classification	Compound name	RT	RI	Formula	MW (g/mol)	CC Ethanol	CC Methanol	CC n-Hexane	Known activity
Alkanes	Dodecane	10.380	200	$C_{12}H_{26}$	170.33	2.17	-	5.27	Enhances antifungal activity (Stopiglia et al., 2012)
	Tetradecane	13.149	236	$C_{14}H_{30}$	198.39	1.49	-	2.36	Antibacterial and antifungal activity (Nasr et al., 2022)
	Hexadecane	15.627	268	$C_{16}H_{34}$	226.44	-	-	3.01	
	Octadecane	17.778	296	$C_{18}H_{36}$	252.5	-	-	2.95	
	Heneicosane	20.798	342	$C_{21}H_{44}$	296.6	1.88	-	2.12	Antimicrobial activity (Vanitha et al., 2020)
	Docosane	21.702	356	$C_{22}H_{46}$	310.6	-	5.09	-	-
	Eicosane	22.564	327	$C_{20}H_{42}$	282.5	18.97	7.19	14.38	Antifungal activity (Ahsan et al., 2017)
	Tricosane	22.567	369	$C_{23}H_{48}$	324.6	-	-	1.84	-
	Tetracosane	22.397	382	$C_{24}H_{50}$	338.7	-	-	2.69	-
	Pentacosane	24.201	394	$C_{25}H_{52}$	352.7	-	-	4.50	-
	Hexacosane	25.068	415	$C_{26}H_{54}$	366.7	-	-	6.09	-
Alkenes	Dodecene	10.262		$C_{12}H_{24}$	160.32	1.24	-	-	-
	1-Tetradecene	13.052	1396	$C_{14}H_{28}$	196.37	3.77	-	-	-
	Cetene	15.540	1587	$C_{16}H_{32}$	224.42	2.51	-	-	-
	9-Octadecene	17.776		$C_{18}H_{36}$	252.5	0.40	-	-	-
	Neophytadiene	18.273	1840	$C_{20}H_{38}$	287.353	8.22	11.02	-	-
	1-Docosene	21.701	2188	$C_{22}H_{46}$	310.6	2.04	-	10.82	-
Benzene	1,4-Di-tert-butylbenzene	11.235	1264	$C_{14}H_{22}$	190.32	3.34	-	4.62	No cytotoxic effect (Jing et al., 2021)
Ester	Methyl salicylate	10.454	1190	$C_8H_8O_3$	152.15	1.75	-	-	Anti-inflammatory activity (Li et al., 2016)
	Benzoic acid, 2,5-bis(trimethylsiloxy)-, trimethylsilyl ester	26.005	1797	$C_{16}H_{30}O_4Si3$	370.66	-	3.59	-	-
Iodinated Hydrocarbons	Docosane, 1-iodo-	14.385	2730	$C_{22}H_{45}I$	436.5	-	-	1.22	-
	1-Iodotriacontane	17.366		$C_{30}H_{61}I$	548.7	-	-	1.97	
	Dotriacontane, 1-iodo-	19.141	3762	$C_{32}H_{65}I$	576.8	-	-	2.85	-
	Octacosane, 1-iodo-	21.167	3354	$C_{28}H_{57}I$	520.7	-	-	2.00	-
	Hexadecane, 1-iodo-	26.081	2064	$C_{16}H_{33}I$	352.34	-	-	5.09	-
Lactones	Dihydroactinidiolide	15.097	1535	$C_{11}H_{16}O_2$	180.24	-	2.87	-	Antioxidant and antiaggregant activity (Das et al., 2018)

Classification	Compound name	RT	RI	Formula	MW (g/mol)	CC Ethanol	CC Methanol	CC n-Hexane	Known activity
Linoleic Acids Ester	Methyl linoleate	20.805	2092	$C_{19}H_{34}O_2$	294.5	-	1.90	-	-
	Ethyl linoleate	21.410	2159	$C_{20}H_{36}O_2$	308.5	-	-	2.29	-
Organosiloxane	Cyclomethicone 7	14.328	1447	$C_{16}H_{21}O_7Si_7$	519.07	4.01	-	-	Antifungal activity (Abdelaziz et al., 2023)
Others	3-Formyl-N-methyl-9-[phenylethynyl]dibenzo [2,3-a : 5,6-a'] (1,4)-thiazine	17.944	-	$C_{22}H_{15}NOS$	341.43	17.05	-	-	-
	3,8-Dimethyldecane	8.223	1140	$C_{12}H_{26}$	170.33	1.42	0.53	-	-
	Unknown	18.065	-	-	-	-	6.84	-	-
	Unknown	19.981	-	-	-	-	9.16	-	-
	Unknown	22.305	-	-	-	-	3.90	-	-
	1,3,14,16-Nonadecatetraene	24.049	1924	$C_{19}H_{32}$	260.5	-	-	4.32	-
	Unknown	24.069	-	-	-	2.66	-	-	-
	Unknown	24.642	-	-	-	-	3.52	-	-
	Unknown	26.820	-	-	-	-	-	15.29	-
Palmitic Acids	Methyl palmitate	19.157	1928	$C_{17}H_{34}O_2$	270.5	-	1.66	-	Anti-inflammatory activity (El-Demerdash, 2011)
	Ethyl palmitate	19.821	1993	$C_{18}H_{36}O_2$	284.5	-	-	2.29	Anti-inflammatory activity (El-Demerdash, 2011)
Phenols	2,5-Di-tert-butylphenol	14.696	1514	$C_{14}H_{22}O$	206.32	1.95	-	-	Antioxidant and anti-inflammatory activity (Zhao et al., 2020)
	Butylated hydroxytoluene	14.701	1511	$C_{15}H_{24}O$	220.35	-	0.93	-	Antioxidant activity (Yehye et al., 2015)
	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	23.485	2398	$C_{23}H_{32}O_2$	340.5	10.2	0.79	3.00	-
Phthalic Acids	Diisobutyl phthalate	18.675	1871	$C_{16}H_{22}O_4$	278.34	3.46	2.71	0.85	Antibacterial activity (Khatiwora et al., 2012)
Polycyclic aromatic hydrocarbon	6-Aza-5,7,12,14-tetrathiapentacene	15.328	-	$C_{17}H_9NS_4$	355.5	6.03	5.91	-	-
Terpenes	Phytol	20.987	2122	$C_{20}H_{40}O$	296.5	-	2.53	-	Antioxidant and antimicrobial activity (Islam et al., 2018)
	Beta-Elemene	26.816	1391	$C_{15}H_{24}$	204.35	-	12.05	-	Anticancer activity (Li et al., 2013)
Thiazoles	2-Acetylthiazole	15.501	1013	C_5H_5NOS	127.17	-	0.91	-	-

RT: Retention time, RI: Retention Index, MW: Molecular Weight, CC: *Calliergonella cuspidata*, "-" Activity not researched; http://www.chemspider.com/; https://pubchem.ncbi.nlm.nih.gov/

3.3. DPPH scavenging activity

C. cuspidata ethanol extract's antioxidant potential was evaluated based on its ability to neutralize stable free DPPH radicals. In this study, the results of the antioxidant potential of *C. cuspidata* ethanol extract obtained at different concentrations (7.8125 to 1000 µg/ml) are provided in Table 2. Since the amount of substance was insufficient, the study was carried out only with ethanol extract. Experimental results showed that all concentrations of *C. cuspidata* ethanol extract remained below 50% as DPPH scavenging activity. However, the increase in extract concentration corresponded to an increase in DPPH scavenging capacity. Ascorbic acid at a concentration of 1 mg/ml, which was the standard used in the experiment, showed a DPPH scavenging activity of 94.66%. The EC_{50} value for ascorbic acid was determined as 0.0455 mg/ml. For *C.cuspidata* the EC_{50} value was determined as 1.0237 mg/ml. When DPPH scavenging rate and extract concentration were evaluated, the correlation value was found to be 0.8957.

Table 2. DPPH radical scavenging activity results of *C. cuspidata* ethanol extract and ascorbic acid (%) with mean ± SD.

Concentration (µg/ml)	***C. cuspidata***	**Ascorbic acid**
1000	47.52 ± 0.90	94.66 ± 0.02
500	39.54 ± 1.54	93.39 ± 0.06
250	36.63 ± 0.47	92.07 ± 0.11
125	34.83 ± 0.35	90.08 ± 0.05
62.5	33.17 ± 0.00	69.94 ± 0.05
31.25	31.79 ± 0.64	35.79 ± 0.08
15.625	30.08 ± 0.59	17.69 ± 0.19
7.81	26.84 ± 0.00	8.739 ± 0.18

4. Discussion

The biologically active substances present in plant extracts are directly linked to a number of actions, including anticancer, antibacterial, antifungal, and antioxidant properties. Via various pathways, these secondary metabolites demonstrate antibacterial and antioxidant qualities. Research has demonstrated that bryophytes generate a number of secondary metabolites that strengthen these fragile plants' robust antioxidant systems and increase their capacity to withstand biotic and abiotic challenges (Dey ve De, 2012). Research results regarding the biological characteristics of *C. cuspidata* moss are limited in the literature. Therefore, biochemical content screening and antioxidant activity determination were conducted in this study on ethanol, methanol, and n-hexane extracts obtained from *C. cuspidata*.

One of the important *Enterobacteriaceae*, *Klebsiella pneumoniae*, is regarded as an opportunistic pathogen that causes a wide range of illnesses and frequently develops drug resistance. It is especially hazardous in hospitals, as it can lead to several serious diseases. It is quite concerning that the isolation rate of *K. pneumoniae* keeps rising (Effah et al., 2020). There is a study indicating the antimicrobial effect of *C. cuspidata* ethanol extract against *K. pneumoniae*. When we look at the content of ethanol extract, we encounter Diisobutyl phthalate, which is known for its antimicrobial effect. A few reports exist on the presence of phthalates in plants. In fact, in one study, dibutyl phthalate was isolated and characterized from *Begoniaceae*. The antimicrobial activity of the isolated compound was found remarkable. Additionally, another study has identified the antimicrobial effect of purified Diisobutyl phthalate (Altuner et al., 2014; Roy et al., 2006; Shobi and Viswanathan, 2018). It is likely that the presence of this compound detected in the ethanol and methanol extracts contributed to the antimicrobial effect.

In another study, the antimicrobial activity was tested with ethanol, methanol, ethyl acetate, and acetone extracts on 13 different strains. While results were observed against all strains, the highest outcomes were exhibited by methanol and acetone extracts against *Escherichia coli* ATCC 11230 (Uyar et al., 2016). It has been proven that *E. coli* is a normal species of bacterium that inhabits the gastrointestinal tracts of warm-blooded animals like reptiles and mammals. Nevertheless, several extremely adaptive commensal clones developed into extremely virulent and often fatal pathogens. Scientists now know that pathogenic *E. coli* and its various pathotypes are linked to a wide range of intestinal and extraintestinal illnesses in humans and animals (Peng et al, 2024). Phytol, which was detected in the methanol extract and whose antimicrobial effect was proven in a previous study, may have caused this result and maybe a drug raw material that can be used in the treatment of *E. coli* pathogens (Islam et al., 2018).

The pathophysiology of chronic illnesses like cancer, diabetes, neurodegenerative disorders, and cardiovascular diseases is significantly influenced by oxidized free radicals, which are known to contribute to a variety of degenerative diseases. Thus, finding potent natural antioxidants is essential to preventing the beginning of degenerative diseases and aging (Sharifi-Rad et al., 2020). Mosses are seen as a potential resource in this regard, but there isn't a single study about this species' antioxidant properties in the literature.

The results of this investigation indicate that the ethanol extract of *C. cuspidata* has DPPH radical scavenging ability that is similar to that of ascorbic acid, the positive control. The EC_{50} value for ascorbic acid was determined as 0.0455 mg/ml. For *C.cuspidata* the EC_{50} value was determined as 1.0237 mg/ml. There is a significant chance of error in this estimate because EC_{50} is outside the observation range. Although it showed less antioxidant activity compared to ascorbic acid, it exhibited better antioxidant activity than ascorbic acid at its lowest concentrations of 0.015625 and 0.0078125 mg/ml. Considering the long storage time of moss, this may have caused the effect to decrease. However, the correlation value of 0.8957 is evidence that the effect may increase if the concentration is increased.

The result of GC-MS analysis of mosses revealed the presence of several compounds with known biological activities that could possibly contribute to the observed antioxidant activity. In the ethanol extract, 3-Formyl-N-methyl-9-[phenylethynyl]dibenzo [2,3-a: 5,6-a'] (1,4)-thiazine and Eicosane were detected. Eicosane, one of the major ingredients, is known for its antifungal activity (Ahsan et al., 2017). Methyl Salicylate (1.75%) is recognized for its anti-inflammatory properties. Cyclomethicone 7 (4.01%) exhibited antifungal activity, while Diisobutyl phthalate (3.46%) demonstrated antibacterial activity (Li et al., 2016; Abdelaziz et al., 2023; Khatiwora et al., 2012).

In the methanol extract, Beta-Elemene and Neophytadiene were identified. Beta-Elemene (12.05%), one of the major components, is a significant compound with proven anticancer effects (Li et al., 2013). Additionally, its compound Dihydroactinidiolide (2.87%) is noteworthy for its antioxidant and antiaggregant activities, while Methyl palmitate (1.66%) exhibits anti-inflammatory activities (Das et al., 2018; El-Demerdash, 2011).

In the n-hexane extract, predominantly alkanes were encountered, but no major compound was detected. Eicosane (14.38%), heneicosane (2.12%) and Tetradecane (2.36%) are compounds known for their antimicrobial properties (Nasr et al., 2022; Ahsan et al., 2017; Vanitha et al., 2020).

5. Conclusion

When comparing the tables, the results of the GC-MS analysis revealed high levels of Eicosane (18.97%), a compound with antifungal properties commonly encountered, Beta-Elemene (12.05%) with anticancer activity, and Dodecane (5.27%) with antifungal bioactive compound. These compounds, isolable from *C. cuspidata* with identified content, can offer numerous benefits in industries, biotechnology, and the health sector.

Declaration

Author contributions: Idea/Concept, DT, SDB, CY, GG, AB, KC; Conceptualization and design, DT, AB, KC; Auditing consulting, DT, SDB, AB; References: ADB, CY, GG, AB; Materials, DT, SDB, CY; Data collection and/or processing, DT, SDB, CY, GG, AB; Analysis and/or interpretation, AB, KC, DT; Literature search, DT, ADB, CY, GG, AB; Writing phase, DT, AB, KC; Critical review, AB, KC.

Conflict of interest: The authors have no competing interests to declare regarding the content of this article.

Funding: The authors declare that no funding, grant, or other support was received during the preparation of this article.

Ethical approval: This research did not involve human or animal subjects and therefore does not require ethical approval.

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