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Phytochemical analyses of *Ebenus haussknechtii* flowers: Quantification of phenolics, antioxidants effect, and molecular docking studies

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ABSTRACT

Plants have been benefited as medicine and food since ancient times. After the discovery of spectroscopy, bioactive compounds in plants have been elucidated and have been utilized in drug development. *Ebenus haussknechtii* has been utilized for traditional medicine. In this study, *Ebenus haussknechtii* flowers were extracted in methanol and quantification of phenolics of this extract was conducted by LC-MS/MS. Antioxidant effect of *E. haussknechtii* flowers was carried out using DPPH free radical scavenging assay, ABTS radical cation scavenging assay, and hydroxyl radical scavenging assay. Quantitative analysis revealed that shikimic acid (0.77 mg/g extract), protocatechuic acid (0.61), catechin (0.34), hydroxybenzaldeyde (0.32) were determined as major products. Hence, the interaction of shikimic acid and DNA gyrase enzyme was calculated theoretically. Moreover, MolDock score, and binding affinity were determined as -73.64 and -5.5 kcal/mol respectively. *Ebenus haussknechtii* flowers displayed good antioxidant activity. In DPPH assay, the extract displayed good activity with the value of 7.27 ± 0.173 (IC_{50}, µg/mL). Moreover, the flower extract exhibited the outstanding ABTS activity with a value of 6.62 ± 0.23 (IC_{50}, µg/mL) in comparison to the extract BHA (7.58 ± 0.15, IC_{50}, µg/mL).

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Ebenus haussknechtii çiçeklerinin fitokimyasal analizleri: Fenoliklerin miktarının belirlenmesi, antioksidan etkisi ve moleküler yerleştirme çalışmaları

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ÖZET

Bitkilerden eski çağlardan beri ilaç ve gıda olarak yararlanılmaktadır. Spektroskopinin keşfinden sonra bitkilerde bulunan biyoaktif bileşikler aydınlatılmış ve ilaç geliştirmede kullanılmaya başlanmıştır. *Ebenus haussknechtii* geleneksel tıpta kullanılmaktadır. Bu çalışmada, *Ebenus haussknechtii* çiçekleri metanol içerisinde ekstrakte edilmiş ve bu ekstraktın fenoliklerinin miktar tayini LC-MS/MS ile yapılmıştır. *E. haussknechtii* çiçeklerinin antioksidan etkisi, DPPH serbest radikal giderme testi, ABTS radikal katyon giderme testi ve hidroksil radikal giderme testi kullanılarak gerçekleştirildi. Kantitatif analizde şikimik asit (0,77 mg/g ekstrakt), protokatekuik asit (0,61), kateşin (0,34), hidroksibenzaldeidin (0,32) ana ürünler olduğu belirlendi. Böylece şikimik asit ile DNA giraz enziminin etkileşimi teorik olarak hesaplandı. Ayrıca MolDock skoru ve bağlanma afinitesi sırasıyla -73,64 ve -5,5 kcal/mol olarak belirlendi. *E. haussknechtii* çiçekleri iyi antioksidan aktivite sergiledi. DPPH testinde ekstrakt 7.27 ± 0.173 (IC_{50}, µg/mL) değeriyle iyi aktivite sergiledi. Ayrıca çiçek ekstraktı, BHA (7.58 ± 0.15, IC_{50}, µg/mL) ile karşılaştırıldığında 6.62 ± 0.23 (IC_{50}, µg/mL) değeriyle olağanüstü ABTS aktivitesi sergiledi.

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1. INTRODUCTION

The use of plants for medicine and food purposes goes back as far as human history, but with the improvement of spectroscopy in the 19th century, plants have become a subject of science (Cragg et al., 1997; Sahin Yaglioglu et al., 2013; Topçu et al., 1999). After this development, many bioactive compounds have been isolated from plants and elucidated, and their biological activities have been investigated (Aksit et al., 2014; Aydin et al., 2016; Elmastas et al., 2016). This situation attracted the attention of synthetic chemists, and they succeeded in synthesizing many natural compounds and modified natural compounds (Cakmak et al., 2006; Erenler et al., 2005, 2007; Erenler et al., 2004). They also increased the effectiveness of natural compounds by functionalizing them (Lu et al., 2014; Ökten et al., 2013). Thus, the rapidly developing pharmaceutical industry had the opportunity to renew itself and develop new drugs to combat various diseases.

The plants include primary and secondary metabolites (Erenler, Atalar, et al., 2023; Y. B. Karan et al., 2024; Cennet Yaman et al., 2024). Primary metabolites are essential compounds produced by plants for basic life functions. These metabolites are involved in processes such as growth, development, and energy making. Secondary metabolites, on the other hand, are not directly involved in basic metabolic processes but often play crucial roles in ecological interactions, defense mechanisms, and signaling. They often have pharmaceutical, agricultural, or industrial significance (Guemidi et al., 2024; Khodja et al., 2023).

Free radicals are highly reactive molecules (T. Karan et al., 2024). This electron configuration makes them unstable and highly reactive, as they seek to gain stability by donating or accepting electrons from other molecules, causing a chain reaction of oxidative damage (Gecer et al., 2023). Free radicals can be generated within the body as part of normal metabolic processes, such as during cellular respiration or immune response (Erenler, Karan, & Bozer, 2023). They can also be produced in response to external factors such as exposure to ultraviolet radiation, pollution, cigarette smoke, and certain chemicals (Erenler & Hosaflioglu, 2023). Antioxidants, which can neutralize free radicals by donating electrons without becoming reactive themselves, play a crucial role in mitigating oxidative stress and preventing cellular damage (Erenler, Chaoui, et al., 2023; Erenler, Gecer, et al., 2023). A diet rich in antioxidant-containing foods, such as fruits, vegetables, nuts, and seeds, can help combat the harmful effects of free radicals and promote overall health and well-being (Atalar et al., 2023; Dag et al., 2023). Antioxidants are molecules that neutralize harmful free radicals (Karan et al., 2022; Sahin Yaglioglu et al., 2022; C Yaman et al., 2022).

Ebenus haussknechtii has been utilized for traditional medicine. *Ebenus* species were reported to display significant biological activities including antifungal, antibacterial, antioxidant, and anticonvulsant. In Turkey, *Ebenus* species were distributed around the Mediterranean and Anatolia regions (Hayta et al., 2014).

Herein, methanol extract of *Ebenus haussknechtii* flowers was prepared, and a quantitative analysis of phenolic compounds was carried out. Moreover, the antioxidant activity of this extract was conducted. Shikimic acid was determined as a major product, so a molecular docking study was executed on this compound.

2. MATERIAL and METHODS

Plant material

Ebenus haussknechtii was collected from Bingol in July 2022 and was identified by Dr. Lutfi Behcet, Bingol University, a voucher specimen was deposited in the herbarium at the same university (No: 20798).

Extraction

Ebenus haussknechtii leaves (5.0 g) were macerated with methanol (120 mL) for 24 hours at room temperature. After filtration, the solvent was evaporated by reduced pressure to yield the crude extract (0.5 g) (Houari et al., 2022).

LC-ESI-MS/MS analysis

The leaves of Ebenus haussknechtii were subjected to LC-MS/MS analysis to quantify bioactive compounds (Agilent Technologies 1260 Infinity II). *Ebenus haussknechtii* leaf extract (50 mg) in Eppendorf was mixed with methanol (1.0 mL). The hexane was added and centrifuged at 10000 rpm for 15 minutes. The methanol phase (100 µL) was diluted by adding water (450 µL) and methanol (450 µL). After filtration (0.22 µm filter), the solution was injected into the device. Formic acid (0.1%) and ammonium formate (5.0 mM) in water A, formic acid (0.1%) and ammonium formate (5.0 mM) in methanol B were used as the mobile phase. The gradient program was set as 20% for 1-5 min, 55% for 6-15 min, 85% for 16-25 min and 5% for 25-30 min for mobile phase B. The injection volume was 5.12 µL and the flow rate was 0.40 mL/min. The capillary voltage was 4000 V and the column temperature was 40°C. 39 standard compounds were used for the analysis (Figure 1) (Erenler, Karan, & Hosaflioglu, 2023).

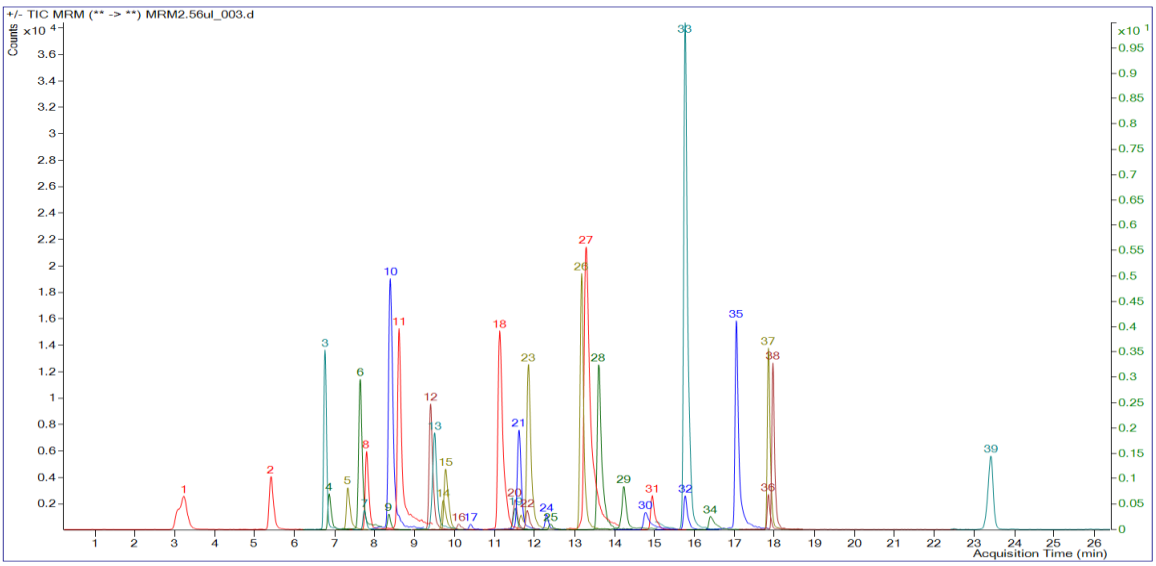

Figure 1. The MRM chromatogram of 39 standard compounds by using LC-MS/MS (1-Gallic acid, 2-Protocatechuic acid, 3-Epigallocatechin, 4-Catechin, 5-Chlorogenic acid, 6-*4*-Hydroxybenzaldehyde, 7-Vanillic acid, 8-Caffeic acid, 9-Syringic acid, 10-Caffeine, 11-Vanillin, 12-*p*-Coumaric acid, 13-Salicylic acid, 14-Taxifolin, 15-Resveratrol, 16-*trans*-ferulic acid, 17-Sinapic acid, 18-Scutellarin, 19-*o*-Coumaric acid, 20-Coumarin, 21- Protocatechuic ethyl ester, 22- Rutin, 23- Isoquercitrin, 24- Hesperidin, 25- Quercetin-*3*-*D*-xyloside, 26- Kaempferol-*3*-glucoside, 27- Fisetin, 28- Baicalin, 29- *trans*-Cinnamic acid, 30- Quercetin, 31- Naringenin, 32- Hesperetin 33- Morin, 34- Kaempferol, 35- Baicalein, 36- Luteolin, 37- Biochanin A, 38- Chrysin, 39-Diosgenin).

DPPH free radical scavenging assay

The samples at different concentrations (4-60 µg/mL) from stock solution were treated with DPPH$^{\bullet}$ solution in ethanol (1.0 mL, 0.26 mM) and then vortexed. The mixture was incubated at rt for 30 min. An absorbance measurement was performed with a spectrophotometer (517 nm). The results were calculated as IC_{50} (Gecer & Erenler, 2022).

$ABTS^{\bullet+}$ radical cation scavenging assay

The stock solution of samples (0.25 mg/mL) were prepared, and phosphate buffer was made then the reaction was carried out in this buffer solution. The different concentration of samples (4-60 µg/mL) was reacted with $ABTS^{\bullet+}$ solution. The measurement was executed by a spectrophotometer (734 nm). The results were calculated as IC_{50} (Gecer, Erenler, et al., 2022).

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was carried out using the *E. haussknechtii* flowers. The hydrogen peroxide (1.0 mL, 40 mM) was mixed with the phosphate buffer (40 mM, 2.4 mL, pH 7.4), and then the sample solution (100 µL) was added to the buffer solution and incubated for 10 minutes. Absorbance measurement was carried out (230 nm) (Erenler, Yaman, et al., 2023).

Molecular docking application

The drawing, 3D structures, and minimum energy of the shikimic acid were calculated in the ChemDraw software. The enzymes chosen for this docking study were DNA gyrase [PDB ID: 1KZN] in the Protein Data Bank. Molecule-enzyme interactions were observed with the Molegro Virtual Docker (MVD) program. Images of interactions (2D, 3D) were captured with BIOVIA Discovery Studio Visualizer. Additionally, binding affinity was calculated with the AutoDock Vina program (Başar et al., 2024).

Statistical analysis

The statistical analysis was conducted by GraphPad Prism (version 8.00). One-way ANOVA followed by Tukay multiple comparison test was carried out. The results were expressed as mean values ± standard deviation ($P < 0.05$).

3. RESULTS and DISCUSSIONS

Ebenus haussknechtii flowers were extracted in methanol, after the removal of methanol the crude extract was yielded. The quantitative analysis of bioactive compounds in methanol extract was carried out by LC-MS/MS. Shikimic acid (0.77 mg/g extract), Protocatechuic acid (0.61 mg/g extract), catechin (0.34 mg/g extract), Hydroxybenzaldeyde (0.32 mg/ g extract) were determined as major products (Table 1).

Table 1. Quantitative analysis of natural compounds in *Ebenus haussknechtii* flowers by LC-MS/MS (mg/g extract)

No	Compound	RT	Amount
1	Shikimic acid	1.2654	0.773
2	Gallic acid	3.4401	0.232
3	Protocatechuic acid	5.4826	0.612
4	Catechin	6.9819	0.343
5	Chlorogenic acid	7.3728	0.143
6	Hydroxybenzaldeyde	7.7280	0.321
7	Vanillic acid	7.8442	0.227
8	Syringic acid	8.2417	0.112
9	Vanillin	8.4197	0.32
10	o-coumaric acid	9.2680	0.45
11	Hesperidin	11.8041	0.332
12	Isoquercitrin	11.9065	0.213
13	Fisetin	13.2761	0.003
14	Luteolin	17.9513	0.144

Abbreviation: nd: not detected, RT: retention time.

Ebenus haussknechtii includes naturally occurring compounds that are significant for pharmaceuticals and food. Shikimic acid is existed commonly in plants due to the intermediate of the shikimic acid pathway. Shikimic acid serves as a crucial starting material in the synthesis of oseltamivir, which is a widely used antiviral medication for treating and preventing influenza infections. Shikimic acid is obtained by certain plants, chemical synthesis, and microbial fermentation. (Ghosh et al., 2012). Protocatechuic acid is a kind of phenolic acid. The presence of protocatechuic acid in pigmented onion scales helps to inhibit the growth of *Colletotrichum circinans*, thereby protecting the onion plants from the detrimental effects of the fungal disease. This natural defense mechanism highlights the importance of phytochemicals in plant health and disease resistance, and it underscores the potential applications of such compounds in agriculture for disease management and crop protection (Kakkar et al., 2014).

Ebenus haussknechtii flowers were found to have a considerable antioxidant effect. In DPPH free radical scavenging activity, this extract displayed the same activity (7.27 ± 0.173, IC_{50}, µg/mL) with the standard BHA (7.10 ± 0.55, IC50, µg/mL). In ABTS cation radical scavenging effect, the same trend was observed. The flower extract revealed excellent activity with a value of 6.62 ± 0.23 (IC50, µg/mL) in comparison to the extract BHA (7.58 ± 0.15, IC_{50}, µg/mL). The flower extract displayed a lower hydroxyl radical scavenging activity (11.53 ± 0.35, IC_{50}, µg/mL) than that of the standard BHA (8.58 ± 0.15, IC_{50}, µg/mL) (Figure 2).

There is an accord between the present study and the reported work. The antioxidant activity of *Althaea officinalis* flowers was investigated and these flowers displayed a good antioxidant effect. (Elmastas et al., 2004). The flavonoids were isolated from *Allium vineale* and they revealed great antioxidant activity (Demirtas et al., 2013). Another study was conducted on *Echinops orientalis* and the compounds isolated from this plant demonstrated a high antioxidant effect (Erenler et al., 2014). The mint genotypes were reported to show considerable antioxidant effects. (Elmastaş et al., 2015). The bioactive compounds were isolated and identified from *Echinacea purpurea* and *Echinacea pallida* and these compounds displayed excellent antioxidant activity (Erenler et al., 2015).

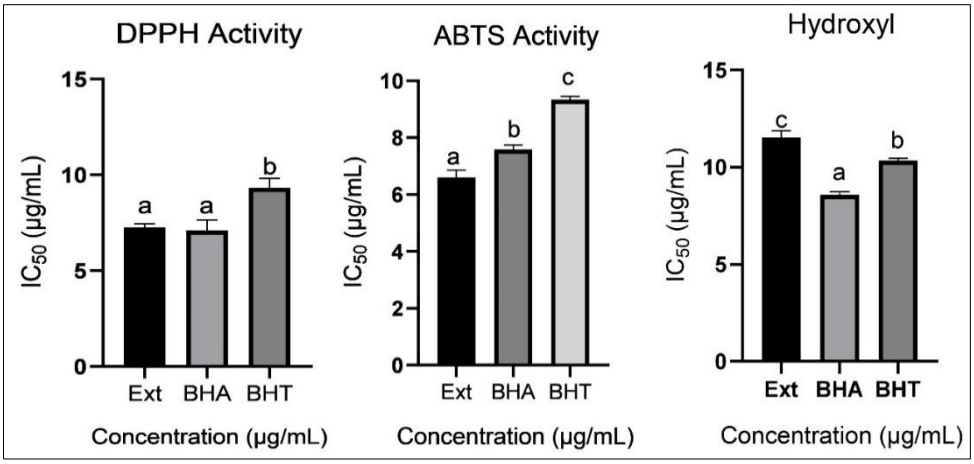

Figure 2. Antioxidant activity of *Ebenus haussknechtii* flowers. Means (three replicates) followed by different letters (a, b, and c) express a statistical difference ($P < 0.05$).

Due to the major compound of shikimic acid of *Ebenus haussknechtii* flowers, the interaction of shikimic acid and DNA gyrase enzyme was calculated theoretically.

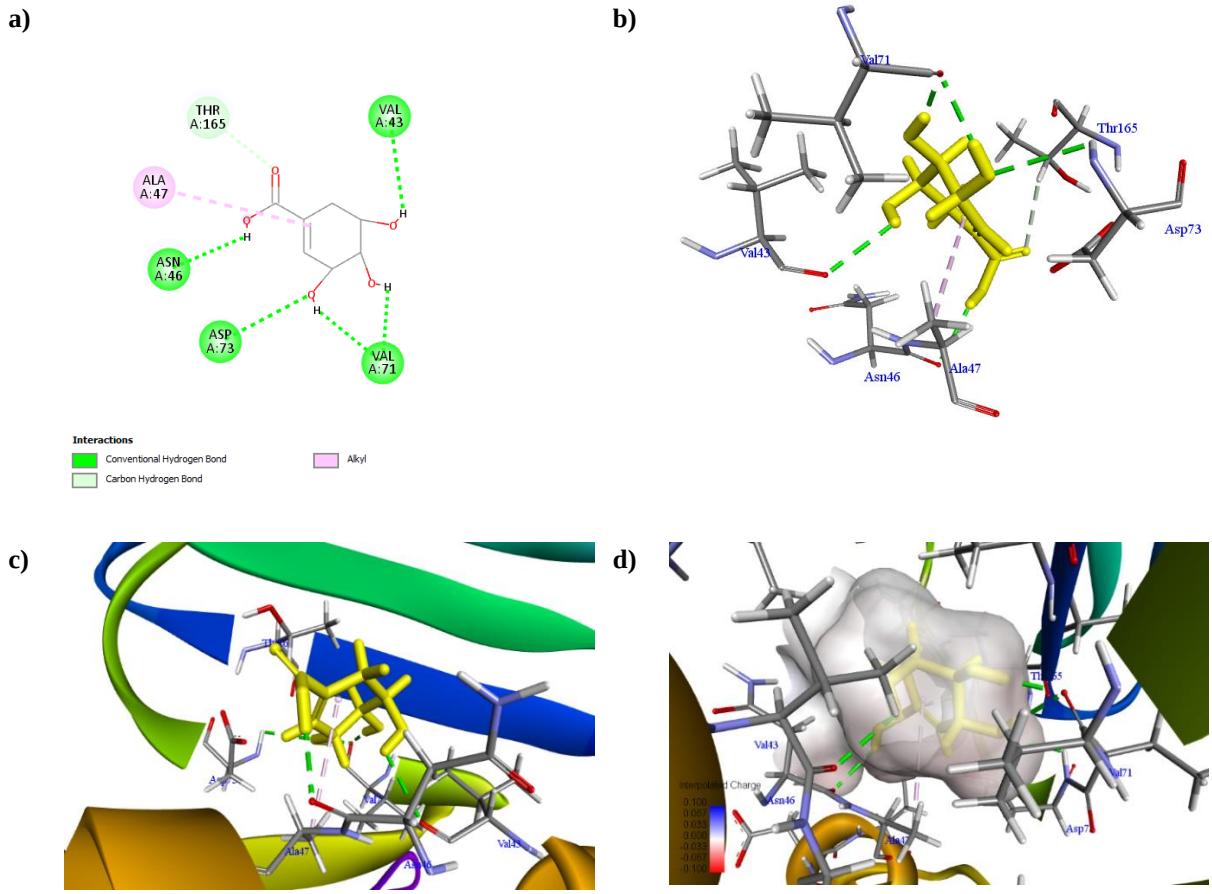

Figure 3. Shikimic acid interaction with DNA gyrase, a) 2D images b) general view c) 3D images d) interpolated load view

Shikimic acid interacted with DNA gyrase by five conventional hydrogen bonds with amino acids ASP73, ASN46, VAL71, and VAL43, one carbon-hydrogen bond with amino acid THR165, and one alkyl with amino acid ALA147 (Figure 3, Table 2). Shikimic acid with DNA gyrase was calculated as a MolDock score of -73.64, with binding energies of -5.5 kcal/mol.

Table 2. Interaction categories, types, and distances of molecular insertion of the shikimic acid molecule with DNA gyrase

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:ASP73:HN - :[001:O4	3.0128	Hydrogen Bond	Conventional Hydrogen Bond	A: ASP73:HN	H-Donor	:[001:O4	H-Acceptor
2	:[001:H7 - A:ASN46:O	2.28384	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H7	H-Donor	A: ASN46:O	H-Acceptor
3	:[001:H8 - A:VAL71:O	1.77862	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H8	H-Donor	A: VAL71:O	H-Acceptor
4	:[001:H9 - A:VAL71:O	2.14128	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H9	H-Donor	A: VAL71:O	H-Acceptor
5	:[001:H10 - A:VAL43:O	2.66703	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H10	H-Donor	A: VAL43:O	H-Acceptor
6	A:THR165:HB - :[001:O2	2.92677	Hydrogen Bond	Carbon Hydrogen Bond	A: THR165:HB	H-Donor	:[001:O2	H-Acceptor
7	A:ALA47 - :[001	4.69407	Hydrophobic	Alkyl	A:ALA47	Alkyl	:[001	Alkyl

4. CONCLUSION

Quantitative analysis of phenolic compounds of *Ebenus haussknechtii* flowers was conducted and shikimic acid was found as a major product. *Ebenus haussknechtii* displayed outstanding antioxidant activity, so, this plant has the potential to be used in food and pharmaceuticals. The theoretical study presented that shikimic acid which is the major product has an inhibition effect on DNA gyrase.

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