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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

In Vitro Biological Activities of *Ranunculus gracilis* Clarke Rhizome

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Abstract: *Ranunculus gracilis* rhizomes were picked from Yenice Forests Karabük province in Turkey. Ethanol was chosen for extraction solvent. Disc diffusion method including filamentous and non-filamentous Gram-positive bacteria, Gram-negative bacteria, and yeast strains was used to evaluate the antimicrobial activity of the extract. 2,2-diphenyl-1-picrylhydrazyl **(**DPPH) free radical scavenging activity and ferric reducing antioxidant power analysis were performed to determine antioxidant activity. The Folin-Ciocalteu method for determining the total phenolic amount and the $AlCl_3$ method for the total flavonoid content of the extract was chosen. Mean diameters of inhibition zones (IZD) of the bacteria were found in the range of 8.2 mm to 24.45 mm. This value was measured as 17.82 mm and 18.69 mm for yeasts. The total antioxidant activity value of the extract was calculated as 7.08 mg AAE/g extract. The IC50 value was found as 9.097 mg/mL for DPPH free radical scavenging activity. The FRAP value indicated that the reducing power of 1 gram of sample was equivalent to 4.66 µmol of Trolox. The total phenolic content of ethanol extract of *R. gracilis* rhizomes was determined as 0.414 mg GAE/g, while the flavonoid content was calculated as 0.68 mgQE/g. This study is the first report demonstrating the biological activities of *R. gracilis* in the literature. The analyzed ethanolic extract of *R. gracilis* rhizomes demonstrated that the biological activity level could be considered significant according to the obtained results.

Keywords: Antibacterial effect, antifungal activity, antioxidant, pathogenic actinomycetes.

Ranunculus gracilis Clarke Rhizomunun In Vitro Biyolojik Aktiviteleri

Öz: *Ranunculus gracilis* rizomları Türkiye'nin Karabük ili Yenice Ormanlarından toplanmıştır. Ekstraksiyon çözücüsü olarak etanol seçilmiştir. Ekstraktın antimikrobiyal aktivitesinin değerlendirilmesinde filamentli ve filamentli olmayan Gram-pozitif bakteriler, Gram-negatif bakteriler ve maya suşlarını içeren disk difüzyon yöntemi kullanılmıştır. Antioksidan aktiviteyi belirlemek için 2,2-difenil-1-pikrilhidrazil (DPPH) serbest radikal temizleme aktivitesi ve ferrik indirgeyici antioksidan güç analizi yapılmıştır. Toplam fenolik miktarı belirlemek için Folin-Ciocalteu yöntemi ve ekstraktın toplam flavonoid içeriği için $AlCl_3$ yöntemi seçilmiştir. Bakterilerin ortalama inhibisyon zonları (IZD) çapları 8.2 mm ile 24.45 mm arasında bulunmuştur. Bu değer mayalar için 17.82 mm ve 18.69 mm olarak ölçülmüştür. Ekstraktın toplam antioksidan aktivite değeri 7.08 mg AAE/g ekstrakt olarak hesaplanmıştır. DPPH serbest radikal süpürme aktivitesi için IC50 değeri 9.097 mg/mL olarak bulunmuştur. FRAP değeri, 1 gram numunenin indirgeme gücünün 4.66 µmol Trolox'a eşdeğer olduğunu göstermiştir. *R. gracilis* rizomlarının etanol ekstraktının toplam fenolik içeriği 0.414 mg GAE/g, flavonoid içeriği ise 0.68 mgQE/g olarak hesaplanmıştır. Bu çalışma, literatürde *R. gracilis*'in biyolojik aktivitelerini gösteren ilk rapordur. Elde edilen sonuçlara göre, *R. gracilis* rizomlarının analiz edilen etanolik özü, biyolojik aktivite seviyesinin önemli olarak kabul edilebileceğini göstermiştir

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Anahtar kelimeler: Antibakteriyal etki, antifungal aktivite, antioksidan, patojenik aktinomisetler.

INTRODUCTION

For human diseases, medicinal plants have been used for centuries because of their components which have therapeutic value. At the same time, the potential of the chemicals obtained from these plants in the research of new drug discovery cannot be ignored (Nostro et al., 2000). Thanks to its flower diversity, our country has a positive potential in medicinal plant research (Ceylan et al., 2019; Kasapoğlu et al., 2020; Ozturk et al., 2018).

Turkey is one of the leading countries on trade in medicinal and aromatic plants, whereby geographic location, climatic characteristics, plant diversity, agricultural potential and large surface area (Pakdemirli et al., 2021). Due to Turkey's climatic and ecological features, many medicinal and aromatic plants can be collected from nature or cultivated in agricultural areas (Türkiş 2018, Wang et al., 2020). Besides, plant extracts in the food, pharmacology, and cosmetics fields are becoming more common. This increasing demand shows that it is crucial to investigate medicinal plants systematically (Wang et al., 2020).

Ranunculaceae are distributed throughout the northern hemisphere and temperate regions in the southern. In Turkey, this family comprises twenty genera, and eighty-four species and twenty-eight subspecies represent the genera of *Ranunculus*. Twenty-two of them are endemic (Güner et al., 2012). *R. gracilis* Clarke is not an endemic species. The plant naturally spreads in the Balkans and Turkey. It is seen on slopes, scrubs, and fields. Its local name in Turkey is narin yağ çiçeği.

It is reported that some *Ranunculus* species have been used for their health benefits. This family has been used to treat rubefacient, antirheumatic, cough and asthma, urinary infections, intermittent fever commonly (Nazir et al., 2013; Raziq et al., 2020). For instance, in Kazakhstan, *R. grandifolius is* used to treat tuberculosis, and in Jordan, *R. asiaticus is* used in rheumatic treatments (Ryabushkina et al., 2008). Also, it is published that In Umbria, Italy, *R. ficaria*'s rhizome, young leaves, and flower parts are used in teeth and mouth cleaning (Ranfa & Bodesmo, 2017). In Turkey, young leaves and tubers of *R. ficaria* L. subsp. *calthifolius* is consumed as food (Elmas et al., 2017). *R. arvensis* is used as a folk remedy for arthritis, asthma, high fever, gout, and psoriasis in the Far East (Akbulut et al., 2011).

It is known that natural products obtained from medicinal plants are chemically balanced and effective. Compared to synthetic drugs, it is considered to have the most negligible side effects and the least harmful (Bhatti et al., 2015). Due to different environmental conditions, the active substances produced by plants may vary in content and density (Vita et al., 2018). Free radicals can cause some health problems. By completely blocking or reducing the formation of free radicals, antioxidants protect people from their harmful effects and help prevent the development of a wide variety of diseases (Bhatti et al., 2015).

The rhizome is the plant organ where concentrated active ingredients such as macro-micro and essential components are stored (Jabborova et al., 2021; Parzych et al., 2015). On the other hand, these parts can be poisonous. Although the poison is neutralized chiefly by heat treatment, these plants should be used as food and medicine with extreme caution (Elmas et al., 2017).

Our literature review found that species highly related to *R. gracilis* were reported to be used as food and medicine. But studies on *R. gracilis* have remained untouched. In the literature survey, there is neither antimicrobial susceptibility nor antioxidant activity investigation for this plant. Therefore, in this study, the antioxidant and antimicrobial activities of the ethanolic extract of the rhizome part of the *R. gracilis* plant were tried to be determined for the first time.

MATERIAL AND METHOD

Material: *R. gracilis* rhizomes were picked from Yenice Forests Karabük province in Turkey during 2014-2016 (Figure 1). Collected samples were pressed in the field, and the species identification was made by Sevda TÜRKİŞ, using Davis et al., (1988)'s book named "Flora of Turkey and The East Aegean Islands Vol. 10". The voucher specimen was deposited at the herbarium of Ondokuz Mayıs University, Samsun, Turkey (OMUB Herbarium No:8847).

(a) (b)

Figure 1. (a) The photo of *R. gracilis* was taken by Sevda TÜRKİŞ **(b)** The location of Yenice Forest. Ethanol extract of *R. gracilis* was prepared according to the modified methods of Wandscheer et al., (2004) (Çil et al., 2021).

Preparation of extracts: Whole plant parts were cleared of soil residues and dried at room temperature for 2 weeks. In the study, it was preferred to use ethanol as a solvent due to its non-toxicity. The powdered plant material was percolated using 95% ethanol in the ratio of 1:10 (w/v) at room temperature for one night and supernatant was collected. This process was repeated for 3 days, and the supernatant was collected in a separate bottle. Then the whole ethanolic extract was filtered through 125 mm quantitative filter paper discs. The solvent was evaporated

by rotary evaporator under reduced pressure and temperature (30°C). The crude extracts were stored at –20°C until used.

Antimicrobial activity: Since the rhizome part of the *R. gracilis* plant remains underground, we chose to use the pathogenic microorganisms to be used in the study predicting that the extract obtained from the rhizome part may have an effect on soil-borne opportunistic pathogenic organisms. In the study, we studied with actinomycetes, which are opportunistic pathogens found in soil In addition, Gram-positive and Gram-negative hospital pathogens, which are frequently encountered in the literature, were used.

The selected microorganisms and their culture collection numbers are listed in Table 1. The fresh culture of each microorganism was transferred to sterile test tubes containing Brain-Heart Infusion (BHI) broth under aseptic conditions. The bacterial and fungal density in the tube was adjusted to 0.5 and 1 McFarland respectively, using McFarland Densitometry. Six mm diameter sterile blank discs (Oxoid) were placed on agar to load 50 µl of extract solution. Antimicrobial activity tests were performed with the disc diffusion method according to the M100 (2021), M02 (2018), M60 (2020) Clinical and Laboratory Standards Institute (CLSI) procedures.

Table 1. List of microorganisms used in the study.

Microorganism	Culture Collection Number
*Bacillus subtilis**	NRRL B-209^T
Candida albicans+	DSM 1386^T
Escherichia coli※	ATCC®25922^T
*Enterococcus faecalis**	ATCC®19433^T
*Micrococcus luteus*δ	NRRL B-1018^T
*Nocardia abscessus*δ	DSM 44432^T
*Nocardia cyriacigeorgica*δ	DSMZ 44484^T
Proteus vulgaris※	NRRL B-123^T
Saccharomyces cerevisiae+	ATCC®9763^T
Salmonella enterica subsp. *enterica* ※	ATCC®43971^T
*Staphylococcus aureus**	ATCC®6538^T
*Streptomyces murinus*δ	ISP 5091^T

*Non-filamentous Gram-positive bacteria δFilamentous Gram-positive bacteria
※ Gram-negative bacteria +Yeast

A digital caliper measured inhibition zone diameters of different microorganisms to estimate the potency of antimicrobial activity after incubation at 37°C for 24-48 h. The study was conducted in three replicates. The obtained results were the mean of three measurements.

Total phenolic content: The total phenolic content of the rhizome extract was determined using Folin-Ciocalteu (Singleton & Rossi, 1965). In this method, it is essential to spectrophotometrically measure the intensity of the blue color of the complex formed by reducing the phenolic content in the extract to the phosphomolybdic-phosphotungstic component contained in the Folin-Ciocalteu reagent. Using the standard calibration curve prepared with gallic acid, the total phenolic content was determined in terms of gallic acid (GA) equivalent (mg GAE/g extract).

The flavonoid content: The flavonoid content of the extract was determined according to the method used by Arvouet-Grand et al., (1994). According to this method, 1 mL of 2% $AlCl_3$ solution prepared in methanol is mixed with the same volume of extract or various concentrations of quercetin. After 10 minutes, absorbances were measured at 415 nm against the prepared blank, and the total flavonoid content of the sample was calculated as quercetin equivalent (mg QE/g extract).

***Total antioxidant capacity*:** The total antioxidant capacity of the prepared sample was determined spectrophotometrically at 695 nm using the phosphomolybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the studied extract used, resulting in the formation of green-colored phosphate/Mo(V) compound (Prieto et al., 1999). Total antioxidant activity was expressed as ascorbic acid equivalent (mgAAE/g extract).

DPPH free radical-scavenging activity: The rhizome sample's DPPH free radical scavenging efficiency was determined according to the method used by Sànchez-Moreno et al., 1998. For this purpose, different amounts of the extract were combined with the DPPH solution prepared in methanol, and at the end of 30 minutes, absorbances of each tube content nm against methanol were recorded at 517. The scavenging activity (%) values calculated using the following equation for each extract concentration were plotted against the concentration. The SC50 value (the extract concentration that scavenges 50% of the free radicals in the environment) was determined from the graph.

$$\text{Scavenging Activity (\%)} = (ABS_{blank} - ABS_{sammple})/ABS_{blank} \times 100$$

Ferric Reducing Antioxidant Power (FRAP): The FRAP method, based on the principle of reducing of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous coloured form (Fe^{2+}-TPTZ) in the presence of antioxidants, was performed by applying the method previously described by Slinkard and Singleton (1977). The FRAP reagent used for the test containing 2.5 mL of a 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M pH 3.6 acetate buffer is prepared daily and kept in the dark at 37° C. Sufficient amount of extract was combined with FRAP reagent and after 30 minutes incubation at 37°C, all tube contents were recorded at 593 nm, including trolox standards of different concentrations exposed to the same conditions. A calibration curve was drawn with the absorbance values were compared with different concentrations of trolox, and the FRAP value of the sample was calculated as trolox equivalent (µmol TXE/g extract), using the straight-line equation of the curve.

Statistical analyses: Statistical Package for the Social Science Predictive Analytics SoftWare Statistics (SPSS) version 26 was used for statistical analyses in this study. First, the normality of the data was checked. Then it was checked whether the data were homogeneously distributed, and the analysis of variance was started. The results were evaluated in the confidence limit of 0.05.

RESULTS

Antimicrobial activity: This study investigated the antimicrobial effect of *R. gracilis* rhizome were tested on filamentous Gram-positive, non-filamentous Gram-positive, and Gram-negative bacteria and yeast strains. The results obtained using the disk diffusion method, which is the oldest and most popular antimicrobial activity method, are given in Table 2 in alphabetical order.

Table 2. Antimicrobial activities of *R. gracilis* ethanolic extract based on inhibition zone diameters (IZD).

Tested Microorganisms	Microorganism groups※	IZD (mm)	Sf300*	Nystatin (0.5 mg/mL)
B. subtilis	NFG+	20.58±0.9^{bc}	33.98±0.5	NIZ
C. albicans	Y	17.82±0.3^{def}	NIZ	32.89±0.2
E. coli	G-	19.98±0.9^{bc}	36.42±0.6	NIZ
E.faecalis	NFG+	8.2±0.1^{g}	34.56±0.5	NIZ
M. luteus	NFG+	24.45±0.6^{a}	40.99±0.2	NIZ
N. abscessus	FG+	16.7±0.2^{ef}	32.30±0.9	NIZ
N. cyriacigeorgica	FG+	20.23±0.07^{bc}	31.40±0.7	NIZ
P. vulgaris	G-	21.8±0.5^{b}	30.90±0.2	NIZ
S. cerevisiae	Y	18.69±0.3^{cde}	NIZ	30.25±0.5
S. enterica subsp. *enterica*	G-	19.31±0.5^{dc}	24.05±0.2	NIZ
S. aureus	NFG+	16.27±0.7^{f}	29.89±0.3	NIZ
S. murinus	FG+	19.48±0.03^{dc}	31.95±0.5	NIZ

*Oxoid Sulfafurazole Antimicrobial Susceptibility Disks 300µg NIZ: No inhibition zone. ※NFG+: non-filamentous Gram-positive FG+: filamentous Gram-positive G-:Gram-negative Y: Yeast

Normality and homogeneity of the data were checked with Shapiro-Wilk and Levene statistics, respectively. Since the significant value (p) for both tests was greater than 0.05, it was concluded that the data were both normally and homogeneously distributed, and parametric analyzes were started. A One-Way analysis of variance (ANOVA) was used to determine whether there was a statistically significant difference between the groups due to the antimicrobial activity test. According to the results above, at least two microorganism groups were statistically different from each other. The highest inhibition zone diameter value was calculated for *M. luteus* and the lowest one was *E. faecalis* and also *P. vulgaris* and *S. aureus* inhibition zone diameter values were differed statistically significantly from the others $F(11, 24)=91.403$; $p<0.05$ (Figure 2). When the antimicrobial activity results were examined, it was seen that Gram-negative and filamentous Gram-positive bacteria were more sensitive than Gram-positive non-filamentous microorganisms $F(3, 32)=5.385$; $p=0.004$.

Total Phenolic and Flavonoid Contents: Total phenolic amount of the rhizome part of the *R. gracilis* Clarke ethanol extract was calculated as 0.414 mg GAE/g extract according to Folin-Ciocalteu method. The total flavonoid content of the same extract was recorded as equivalent to 0.68 mg quercetin used as standard for 1 g of the extract.

Total Antioxidant Activity: The antioxidant activity of the plant extract, whose phenolic and flavonoid content was revealed, was first evaluated by the phosphomolybdenum method. This method is quite suitable for estimating the antioxidant activity of crude extracts on a total basis. The result was expressed in comparison with the widely used standard antioxidant ascorbic acid and calculated value was 7.08 mg AAE/g extract.

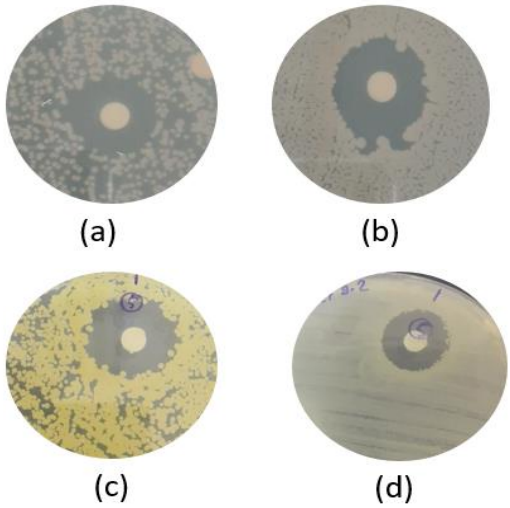

Figure 2. Antimicrobial activity of ethanolic extract of *R. gracilis* rhizome photos was taken by Elif ÇİL. **(a)** *C. albicans* **(b)** *S. enterica* subsp. *enterica* **(c)** *M. luteus* **(d)** *S. aureus*

Antioxidant Activities: Apart from total antioxidant capacity assay, antioxidant activity methods support each other were also carried out. Antioxidant activities correlate the antioxidant power of the tested substance with its ability to transfer electrons. Firstly, the

effect of saving the tested sample from being a radical form by presenting an electron atom to the DPPH radical in the medium was tested. The result was calculated with the SC_{50} value, that is, the concentration of the extract that scavenges half of the radical in the medium. SC_{50} value was obtained as 9.097 mg/mL. The iron ion reducing power, namely FRAP value, known as trolox equivalent and a widely known antioxidant, was also calculated as 4.66 µmol TXE/g extract.

Table 3. Total phenolic and flavonoid contents and antioxidant screening of *R. gracilis* rhizomes.

Assays	Measurement Unit	Values
Total phenolic content	(mg GAE/g extract)	0.414
Total flavonoid content	(mg QE/g extract)	0.68
Total antioxidant activity	(mg AAE/g)	7.08
DPPH	(IC_{50};mg/mL)	9.097
FRAP	(µmol of TXE/g extract)	4.66

DISCUSSION AND CONCLUSION

Although there are some *Ranunculus* species that have been used in Asian traditional medicines, there have been no reports about *R. gracilis* yet. This study is the first report of in vitro biological activity of *R. gracilis* rhizomes in the literature. So, antimicrobial activity and antioxidant assay methods were combined to evaluate the biological activities of the rhizome.

Antibacterial and antifungal activity are two important steps to screen antimicrobial activity. When many studies investigating the antimicrobial activity of an extract in the literature are examined, it has been found that researchers prefer to use only Gram-positive and Gram-negative bacteria such as *S. aureus* and *E. coli* which are frequently encountered in hospitals (Aladesanmi et al., 2019; Masood et al., 2020; Önalan et al 2021; Mulat et al., 2022). There is a need for the discovery of new antimicrobial agents for some filamentous bacteria such as *Nocardia*, which are known as opportunistic pathogens that can cause nosocomial infections in hospitals. In addition to being the first study reported in the literature, the other unique aspect of the study is that it also includes opportunistic pathogens of filamentous actinomycete strains.

In their study conducted in 2018, Atcı and Karagöz compared the antimicrobial activities of *R. sericeus* extracts with methanol and acetone. The inhibition zone diameters they obtained were 15 mm for *B. subtilis*, 13 mm (acetone), 12 mm (methanol) for *E. fecealis*, 11 mm (acetone) and 14 mm (methanol) for *S. aureus*. Except for *E. fecealis*, the inhibition zone diameters obtained in that study were smaller than ours (Table 2). When evaluating the results obtained by the disk diffusion method, the inhibition zone diameter of 15 to 19 mm can be considered as medium, and the inhibition zone diameter of 20 mm and above can be considered as strong antimicrobial activity (CLSI 2018). Therefore, our results can be evaluated on a scale ranging from intermediate to strong, except for *E. facialis*. It is expected result because the rhizome, which is the main structure to be protected in geophyte plant species, remains under the ground. It would not be surprising that these parts produce antibiotic-like secondary metabolites in order to protect them from pathogenic bacteria and the other living things in the soil.

When the literature is examined, it is seen that (same as antimicrobial activity screening), the phenolic and flavonoid contents and their antioxidant activities of the leaves, roots, and flower parts of different *Ranunculus* species except *R. garcilis* are investigated. Therefore, the present study aimed to biochemically investigate the rhizome part of *R. gracilis*, which is thought to be incomplete in the literature.

As a result of this study, it was figured out that the ethanolic extract prepared from the rhizome of the plant had a phenolic content using the gallic acid equivalent and a flavonoid content as well using the quercetin equivalent. Both the DPPH radical scavenging ability and the trolox equivalent FRAP value were examined in addition to the total antioxidant activity test. The obtained values are moderate when compared with similar studies (Deghima et al., 2020). Deghima et al. (2020) investigated antioxidant activity of different solvent fractions from the roots of *R. macrophyllus* Desf and obtained the best results in the ethyl acetate fraction. Similarly, Bhatti et al. (2015), who made post-extraction examinations in different solvent combinations such as single, double or triple, obtained the highest values for *R. arvensis* in case of methanol extract, and the presented values are quite consistent with the present study.

Among the studies on the total phenolic contents and antioxidant activities of the extracts of the leaf, root, and flower parts of *R. laetus* species prepared with different solvents, the methanol extract of the flower part especially stands out (Masood et al., 2020). Such reports common in the literature have led us to study only alcohol extract. The antimicrobial potential of *R. gracilis* rhizome, combined with the promising antioxidant activity detected, makes it a strong candidate for further research to find future drugs where such activity is required. However, the plant parts seemed to deserve other detailed investigations of their individual biologically active components, which may be an attractive source of nutraceuticals and medicinal additives.

In a study, in which the polyphenol profile and antioxidant activities of four different *Ranunculus* species collected from Romania were examined after two different extraction techniques. It was concluded that both the plant part and the extraction solvent created a significant

difference. Especially, the difference in species significantly caused variability in the antioxidant activity and phenolic content (Neag et al., 2017).

Furthermore, the rhizome part of *R. gracilis* seemed to deserve other detailed investigations of their individual biological active components, which may be an attractive source of nutraceuticals and medicinal additives. On the other hand, The fact that the International Union of Conservation of Nature (IUCN) category of the populations of *R. gacilis* in Turkey has not been determined shows our lack of information about the conservation status of this species. Our study emphasizes the biological activity of the species and thus its importance. Although we have obtained promising antimicrobial activity data from *R. gracilis*, it may not be correct to recommend it for pharmacostatic studies. At this step, the population distribution and conservation status of the species in Turkey are unknown because these plants are the genetic heritage we will leave to our future generations.

Authors' Contributions

All authors performed data collection and analysis, discussed the results, and contributed to the final manuscript.

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Statement of Conflicts of Interest

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The authors declare that this study complies with Research and Publication Ethics

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