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The effect of *Thymus vulgaris* essential oil on the formation of *Candida albicans* biofilm on denture base materials: An *in vitro* study

Thymus vulgaris esansiyel yağının protez kaide materyalleri üzerinde *Candida albicans* biyofilm oluşumuna etkisi: *İn vitro* bir çalışma

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Abstract

Aim: This study aims to evaluate the antifungal properties of *Thymus vulgaris* (thyme) essential oil (TVEO) on *Candida albicans*-infested polymethyl methacrylate (PMMA) denture base materials and identify the volatile components of *T. vulgaris* essential oil.

Materials and Methods: The investigation involved establishing *C. albicans*'s presence on resin surfaces manufactured in 1x1x0.1 cm size. The resin surfaces on which the *C. albicans* biofilm had formed were then soaked in a 2% and 5% solution of TVEO and the effect on the amount of *C. albicans* colonisation were evaluated. The volatile components of TVEO were determined using the GC-MS method.

Results: Solutions prepared from 2% and 5% TVEO showed better antifungal activity than Corega. The major components detected by GC-MS in the TVEO were carvone 61.36%, linalool 8.32%.

Conclusion: TVEO, showed significant antifungal effects on PMMA resin surfaces. This oil can, therefore, be recommended as an inexpensive, uncomplicated and efficient natural cleaning agent for those wearing dentures.

Keywords: Antifungal activity; *C. albicans*; Denture cleansers; *Thymus vulgaris*.

Öz

Amaç: Bu çalışmanın amacı *Candida albicans* tutulumu sağlanmış polimetil metakrilat (PMMA) protez kaide materyallerine *Thymus vulgaris* (kekik) esansiyel yağının (TVEO) antifungal etkisini değerlendirmek ve TVEO'nun uçucu bileşenlerini belirlemektir.

Gereç ve Yöntem: Çalışmada ilk olarak 1x1x0,1 cm boyutunda hazırlanan rezin yüzeylerine *C. albicans* tutulumu sağlandı. Daha sonra rezin yüzeyleri, TVEO'dan hazırlanan %2 ve %5 oranındaki solüsyonunda bekletilerek *C. albicans* kolonizasyonundaki değişiklik değerlendirildi. TVEO'nun uçucu bileşenleri GC-MS yöntemi ile belirlendi.

Bulgular: TVEO'dan %2 ve %5 oranında hazırlanan solüsyonlar Corega'ya göre daha iyi antifungal aktivite gösterdi. TVEO'da GC-MS ile tespit edilen majör bileşenler carvone %61,36, linalol %8,32.

Sonuç: TVEO, PMMA rezin yüzeylerinde etkili antifungal aktivite gösterdi. Protez kullanıcıları için alternatif, ucuz, basit ve etkili bir doğal temizleyici olarak önerilebilir.

Anahtar Kelimeler: Antifungal aktivite; *C. Albicans*; Protez temizleyiciler, *Thymus vulgaris*.

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Introduction

Denture stomatitis is a condition that results from oral candidiasis in denture wearers. Denture stomatitis is frequently caused by *Candida albicans*, which attach to the denture base and form a biofilm.[1] To avoid denture stomatitis, meticulous denture washing is essential, and the accumulation of biofilm on the denture surface must be prevented.[2-4] Denture cleaning is performed using mechanical and/or chemical methods.[5] Mechanical brushing, while often used for denture hygiene, can cause wear on the denture base and relining materials and increase surface roughness due to its abrasive nature.[2] Increased roughness promotes infestation with *C. albicans*. In addition, the use of chemical cleaning agents may be more effective and easier than mechanical cleaning in elderly patients due to their weakened manual dexterity.[6] Chemical cleansers often include alkaline peroxides, alkaline hypochlorite, acids, disinfectants, and enzymes.[7]

Polymethyl methacrylate (PMMA) is a versatile, long-lasting, safe, visually pleasing, and practical material that is easy to work with. Furthermore, it has limited permeability and solubility to oral fluids. Because of its physical qualities, it is simple to manufacture and fix.[8,9]

The study investigated *Thymus vulgaris*, a member of the Lamiaceae family, commonly known as thyme. *Thymus* species have strong antibacterial and antifungal properties. *Thymus* essential oil is extracted from the aerial parts of the plant by the water-steam distillation method.[10] *Thymus* essential oils exhibit strong antifungal properties against *C. albicans*. Essential oils consist of volatile compounds such terpenoids, phenylpropanoids, and fatty acids.[11]

In this study, the antifungal effect of the essential oil of *T. vulgaris* on the colonisation of *C. albicans* in heat-polymerised dental restorative material (PMMA) was investigated. This study aims to evaluate the antibacterial efficacy of a denture cleanser formulated with *Thymus* essential oil in comparison to the commercially available Corega denture cleanser.

Materials and Methods

Plant materials

The essential oils of Thyme (Thymus Vulgaris Flower/Leaf Oil) were sourced from Elantra Pharmaceuticals Health Cosmetics Ltd. Sti. Phytoil Aromatherapy brand (Turkey).

Preparation of the denture base material

PMMA resin samples, sized 1x1x0.1 cm, were utilised in the investigation. The samples were polished appropriately and kept in distilled water at 37°C for 48 hours. Roughness values of the samples were measured with a profilometer (Surtronic 25; Taylor Hobson, Leicester, United Kingdom) (0.845±0.2). In this study, the measurement length was set as 2.5 mm and the cut-off value was set as 0.25 mm. The surface roughness value of each sample was calculated by averaging repeated measurements in 3 different areas on the surfaces of the sample. The surface roughness values of the samples were standardized. The samples' average surface roughness values (Ra) were assessed using a prolithometer.[12]

Adhesion of *Candida albicans* to denture base materials

The prepared resins were sterilized by autoclaving before the study. 24-hour-old fresh *C. albicans* (ATCC 10231) strain was used to ensure colonization of resin surfaces with *C. albicans*. Standard suspension of 1-2 x 10^8 CFU (~0.5 McFarland) was prepared in Sabouraud Dextrose Broth (SDB) medium from young cultures of *C. albicans*. Then, the identical resin pieces were kept in this suspension at low shaking speed (120 rpm) for 1 hour to ensure the retention of *C. albicans*. Previous studies and the negative control in our study have shown that 1 hour is sufficient for *C. albicans* to adhere to resin surfaces. At the end of the period, the pieces removed from the suspension were gently washed with sterile isotonic solution (0.9% NaCl) in accordance with aseptic rules.[13-15]

Treatment of resin surfaces with essential oils

Resin surfaces contaminated with *C. albicans* were exposed to *T. vulgaris* essential oil (Elantra Pharmaceuticals, Turkey)

solutions of 2% and 5% diluted in 10% dimethyl sulphoxide (DMSO) for 1 hour in a shaken environment. It was then gently washed once with sterile isotonic solution to remove the essential oil from the resin surfaces. The resins were incubated in 5 mL SDB in a shaking oven for about 18 hours before being transferred to Sabouraud Dextrose Agar. Then, 100 µm of these samples were taken and plated on SDA medium and incubated at 35˚C for 24 hours. The results were analyzed not only by counting the colonies on a solid medium, but also by measuring the absorbance values at 600 nm. Resins treated with Corega (GlaxoSmithKline, Ireland) alone served as a positive control group, while resins that retained *C. albicans* but were not treated with essential oil served as a negative control. Corega cleansing tablet was prepared by placing 1 tablet in 200 mL of water. All treatments and controls were carried out under identical conditions.[13,14]

Gas chromatography-mass spectrometry (GC-MS) and GC-Flame Ionization Detector (GC-FID) analyses

An Agilent system at the Eastern Anatolia High Technology Application and Research Centre (DAYTAM) was used to evaluate the oil by GC-FID and GC-MS. GC-MS analysis was performed using a Shimadzu QP2010 Ultra GC-MS system equipped with an HP-5 MS column; 30 m length x 0.25 mm ID diameter, 0.25 µm film thickness. The GC analysis included an AOC-20i autoinjector, a mass spectrometry detector and an AOC-20s sampler. The carrier gas utilised in the experiment was helium with a flow rate of 1.02 mL/min. The chromatographic analysis was performed with a flow rate of 1.02 mL/min and an injection volume of 1 µL in split mode (20:1). The temperature of the GC oven was kept constant at 60 ºC for 10 minutes, after which it was raised by 4 ºC and kept at a fixed temperature of 220 ºC for a further 10 minutes. The incubation process was carried out at a temperature of 246 ºC for a duration of 1.0 minutes, gradually increasing the temperature by 1.0 ºC per minute. The injector and detector temperatures were set at 250 and 300 ºC, respectively. The MS detector parameters were set as follows: transfer line temperature of 300 °C, solvent delay of 3 minutes, electron energy of 70 eV, and the MS was operated in electron impact mode with selected ion monitoring for quantitative analysis.

Identification of components

The essential oils' components were identified by comparing their mass spectra with the W9N11, FFNSC library and validated by comparing the retention durations with authentic samples.

Statistics

The statistical analyses were conducted using the SPSS version 25.0 software. The variables' normal distribution appropriateness was assessed by histogram graphs and the Kolmogorov-Smirnov test. The presentation of the descriptive analysis included the mean, standard deviation, median, and min-max values. An independent T-test was conducted to analyze the absorption values of essential oils produced at 2% and 5%. Instances with a *p* value less than 0.05 were considered statistically significant.

Results

The absorbance value of 2% concentration of TVEO (0.084 ± 0.002) was lower than the absorbance value of 5% concentration (0.095 ± 0.003). Using the absorbance values of all experimental groups, the percentage of viability [(Absorbance experiment/ Absorbance control)*100] was calculated. Antibiofilm properties at 2% and 5% concentration values prepared for TVEO were compared with control and Corega® and are shown in Figure 1 and Table 1. The viability value of 2% Essential oil concentration was found to be 14.55%, the viability value of 5% Essential oil concentration was 16.46%, and Corega was found to be 18.54%. The viability value of the control group was accepted as 100%. The comparison of the % vitality values between TVEO concentration of 2% and 5% was made and the difference between them was found to be significant ($p<0.05$). Considering the anticandidal activity values, it was determined that TVEO was extremely effective, showing better effectiveness than Corega ($p<0.05$). (Figure 1, Table 1)

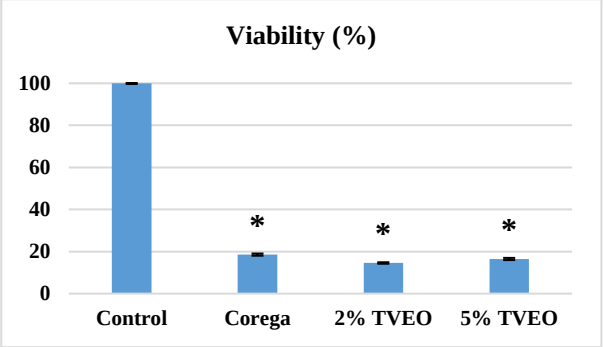

Figure.1. Evaluation of the effect of TVEO (2% and 5%) and Corega on *C. albicans* biofilm in the context of viability% *There is a statistically significant difference with the control group (p<0.05)

There was no colony growth on solid medium after treatment with 2% and 5% of *T. vulgaris* essential oil. An absorbance value of 0.107 (<10^2 CFU/mL) was measured in the resin treated with Corega, which was tested as a positive control in the study. The absorbance value of 0.577 (>10^5 CFU/mL) was measured in the resin tested as a negative control.

Table 1. Absorbance values of Corega and 2% and 5% TVEO

Samples	Absorbances	
	Mean±SD	Medyan (Min-Max)
2% TVEO	0.084±0.002	0.084 (0.082-0.086)
5% TVEO	0.095±0.003	0.095 (0.092-0.098)
Corega	0.107±0.003	0.107 (0.104-0.110)
Control	0.577±0.002	0.577 (0.575-0.579)

TVEO= *T. vulgaris* essential oil, sd=standard deviation
p^1=0.001 Corega vs 2% TVEO
p^2=0.018 Corega vs 5% TVEO
p^3=0.006 2% TVEO vs 5% TVEO

The GC-MS analysis of *T. vulgaris* essential oil revealed the following primary volatile components: carvone 61.36%, linalool 8.32%, thymol 5.44%, and p-cymene 5.18%, a comprehensive content analysis of which is presented in Table 2.

Table 2. Chemical composition (%) of the essential oil of *T. vulgaris*, confirmed by GC-MS

Peak	Name	Retation Time	Area%
1.	Butanoic acid, 2-methyl-, methyl ester	3.42	0.03
2.	*α*-Pinene	6.72	0.48
3.	*β*-fenchene	7.23	0.20
4.	1-Octen-3-ol	8.083	0.27
5.	Octan-3-one	8.351	0.02
6.	Myrcene	8.53	1.04
7.	Ethyl-hexanol	8.64	0.03
8.	*α*-Phellandrene	9.03	0.17
9.	δ-3-Carene	9.25	0.08
10.	*α*-Terpinene	9.47	0.89
11.	**p-Cymene**	9.76	**5.18**
12.	Sylvestrene	9.92	0.52
13.	Eucalyptol	10.02	0.29
14.	*α*-Pinene	10.22	0.04
15.	*β*-Ocimene	10.62	0.06
16.	γ-Terpinene	11.07	1.63
17.	Trans-sabinene hydrate	11.39	0.02
18.	Cis-Linalool oxide	11.60	0.06
19.	Terpinolene	12.26	0.30
20.	**Linalool**	12.73	**8.32**
21.	Trans-γ-Caryophyllene	12.87	0.07
22.	β-Thujone	12.99	0.03
23.	Trans-Pinocarveol	14.38	0.05
24.	(+)-2-Bornanone	14.61	0.10
25.	Borneol	15.53	1.54
26.	L-4-terpineol	16.03	1.59
27.	Cuminic alcohol	16.33	0.11
28.	*α*-Terpineol	16.60	0.49
29.	Thymol methyl ether	18.90	0.22
30.	Nerol	19.60	0.09
31.	Carvenone	20.12	0.05
32.	**Thymol**	21.04	**5.44**
33.	**Carvone**	21.75	**61.36**
34.	Octadecanoic acid, 9,10-dihydroxy-, methyl ester, bis(trifluoroacetate)	21.97	0.25
35.	1,3-Dioxolane, 2,2-dimethyl-4,5-di-1-propenyl-	22.27	0.04
36.	2-Ethyl-5-n-propylphenol	22.34	0.06

37.	Eugenol	23.81	0.03
38.	cis-Geranyl acetate	24.11	0.09
39.	Carvacryl acetate	24.45	0.29
40.	Copaene	24.65	0.05
41.	trans-Geranyl acetate	24.91	0.18
42.	β-Bourbonene	25.04	0.04
43.	Isoeugenol methyl ether	25.78	0.17
44.	Caryophyllene	26.49	1.55
45.	*α-Cis*-Bergamotene	27.11	0.09
46.	Aromadendrene	27.28	0.27
47.	*α*-Humulene	27.87	0.17
48.	γ-Cadinene	28.80	0.04
49.	Ledene	29.56	0.19
50.	β-Bisabolene	30.08	3.16
51.	γ-Cadinene	30.31	0.20
52.	δ-Cadinene	30.66	0.22
53.	trans-α-Bisabolene	31.40	0.06
54.	Spatulenol	32.77	0.21
55.	Caryophyllene oxide	32.99	0.37
56.	Epicubenol	34.20	0.03
57.	δ-Cadinene	35.15	0.36
58.	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	35.36	0.04
59.	3-methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1-pentyn-3-ol	35.78	0.04
60.	Viridiflorol	36.26	0.03
61.	*α*-Bisabolol	36.70	0.05
62.	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	36.81	0.06
63.	Hexahydrofarnesyl acetone	42.29	0.06
64.	3-Benzylsulfonyl-2,6,6-trimethylbicyclo(3.1.1)heptane	45.14	0.42
65.	13-epi-Manoyl oxide	47.16	0.08
66.	5-Isopropyl-2-methylphenyl 3-methylbutanoate	47.97	0.12
67.	5-Androstene, 4,4-dimethyl-	50.41	0.04
68.	2-(7-Hydroxymethyl-3,11-dimethyl-dodeca-2,6,10-trienyl)-[1,4]benzoquinone	50.70	0.05
69.	4-p-Hydroxyphenyl-2,2,4-trimethylchroman	50.96	0.03
70.	Verticiol	51.51	0.08

Discussion

In this study, volatile components were determined by GC-MS analysis of TVEO. It was then investigated whether this essential oil could be used to prevent candida retention in the denture material and as a natural alternative to chemical cleaners used for general cleaning purposes.

Essential oils provide antispasmodic, anti-irritating, antiseptic, antifungal, antiviral, and antibacterial effects. Essential oils have antibacterial and antiseptic qualities that may effectively combat bacteria, molds, and yeasts. The most antiseptic oils include cinnamon, thyme, clove, lavender, and eucalyptus oil. Terpenes being the primary components of essential oils have prompted investigations into the biological characteristics of chemicals within this category. Thymol and carvacrol, which are contained in thyme oil, are 20 times more antiseptic than phenol, for example, and are also utilized in toothpastes. These chemicals possess antioxidant and antibacterial effects.[16] TVEO used in the current study showed antifungal activity by acting on *C. albicans* fungus.

C. albicans is a prevalent pathogen responsible for oral and/or systemic candidiasis. *Candida* species are recognized for their ability to create biofilms on medical surfaces.[17] Fungal infections, however less frequent than bacterial infections, may result in more severe complications. Fungi biofilm structure is resistant to antifungal medications, causing standard antifungal therapy to fail. Hence, there is a need to investigate alternative therapies and develop more effective anticandidal drugs with fewer side effects or

toxicity from various sources, including medicinal plants.[18] Medicinal herbs with essential oils have strong antifungal properties against fungi.[19] *Thymus* species are plants that belong to the Lamiaceae family and are known for their fragrant essential oils and beneficial antifungal and antibacterial properties, which have been widely used since time immemorial.[10] Several *Thymus* species, including *T. kotschyanus*[20], *T. vulgaris, T. zygis, T. satureioides, T. mastichina*[21], *T. capitatus*[22], *T. villous*[23], *T. ciliates*[24], are claimed to have an anticandidal effect. According to the results of the current study, it can be said that *T. vulgaris* essential oil can be used as an alternative anticandidal product.

Aslan et al.[25] conducted a study on the antibacterial and antifungal properties of essential oils. They found that geranium, lemongrass, rosemary, thyme, tea tree, and peppermint oils had inhibitory zones exceeding 5 cm for *C. albicans*. The study found that thyme oil was the most beneficial among the essential oils examined.

Karpinski et al.[26] discovered that peppermint oil (*Mentha piperita*), rosemary oil (*Rosmarinus officinalis*), and thyme oil (*Thymus vulgaris*) showed similar effectiveness against *Candida* species. The study's findings indicate that the essential oils of *T. vulgaris* had the most potent anti-biofilm action, eliminating almost 90% of the biofilm. The oil extracted from *Rosmarinus officinalis* (rosemary) had a diminished efficacy, eliminating around 75.85% of the biofilm. The *in silico* toxicity analysis in this study indicated that the primary chemicals found in the essential oils of plants from the Lamiaceae family are not expected to have carcinogenic, mutagenic, or cytotoxic effects. Nevertheless, it was shown that these oils can cause skin sensitization.[26] In the current study, TVEO showed a strong effect by destroying 83.54% of biofilm at a concentration of 2%.

In the study by Abers et al.[27], *C. albicans*, one of the fungal species, proved to be the most susceptible species to the volatile substances of thyme and rosemary.[27] Fani and Kohanteb[28] conducted a study to assess the antibacterial effects of the essential oil of *T. vulgaris* on certain oral pathogens such as *S. pyogenes, S. mutans, C. albicans, P. gingivalis*, and *A. actinomycetemcomitans*. The study found that *T. vulgaris* oil, at doses ranging from 16 to 256 μg/mL, exhibited a potent inhibitory effect on all clinical isolates using the agar disc diffusion method.

Kavianirad et al.[29] conducted a study to examine the impact of *T. vulgaris* essential oil on *C. albicans* found in removable orthodontic apparatus, utilizing the disc diffusion method. The study found that the essential oil of *T. vulgaris* was substantially more efficient than chlorhexidine (p<0.05) in removing *C. albicans* from the surface of orthodontic apparatus. The study revealed that the essential oil of *T. vulgaris* has superior antifungal properties compared to Corega.

Thyme essential oil demonstrates more effective antifungal activity than black cumin oil and CD Clean®, according to the findings of a study conducted to evaluate and compare the efficacy of thyme essential oil, *Nigella sativa* oil and two commercially available denture cleansers (CD Clean® and Fittydent®) against *C. albicans* adhering to soft denture material.[30]

In a study conducted by Corticchiato et al.[31], it was reported that the essential oil extracted from the plant *Thymus herba-barona* has a concentration of 6.7% carvacrol, 74.6% carvone and 9.5% limonene in its composition. In another study, p-cymene (8.41%), γ-terpinene (30.90%) and thymol (47.59%) were named as the main constituents of the essential oil of *T. vulgaris*.[32] In our study, the essential oil of *T. vulgaris* was found to consist mainly of 61.36% carvone, 8.32% linalool, 5.44% thymol and 5.18% p-cymene (Table 1). When the studies are examined, there are differences in the composition of essential oils, which may be due to harvest location-date, soil and light conditions.[33,34]

Carvone, the first major ingredient in TVEO, is a monoterpene compound that exhibits antibacterial, antifungal, antioxidant, antiepileptic and anticancer effects. Essential oils rich in carvone possess fungicidal and bactericidal effects against a wide variety of pathogenic fungi (e.g. *C. albicans*) and bacteria. According to the findings of several

scientific investigations, essential oils that are abundant in carvone not only inhibit the growth of the *Candida fungus* but also prevent it from converting into its pathogenic form.[35] In a study investigating the fungicidal effect against various *Candida* species (*C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*), carvone was reported to have an antifungal effect.[36]

Linalool is a phenolic volatile compound and is the second major substance in TVEO. Hsu et al.[37] conducted a study demonstrating the antifungal properties of linalool against *C. albicans* (ATCC 14053) and its effects on the development of biofilms. It was also observed to inhibit the development of germ tubes and biofilms in this strain.

Thymol is one of the most important phenolic components found in *Thymus* species. It is also seen as the third major component in TVEO. A study by Braga et al.[38] reports that thymol not only inhibits the formation of *Candida* biofilms on *C. albicans* strains ATCC 3153A and ATCC MYA 2876, but also inhibits the initial stages of biofilm production.

It has also been reported in the literature that carvone, linalool and thymol major components in the *Thymus* essential oil used in our study have a significant anticandidal effect.[36-38] When used together, the volatile components have a synergistic effect that is more effective than when they are used separately.[39] In the study conducted, it is thought that the components in TVEO have a synergistic effect.

Effervescent tablet cleansers produce multiple oxygen bubbles on the denture surface, which stay longer and give a mechanical impact to remove debris and destroy biofilm. This provides a benefit compared to liquid cleansers.[40] The free radicals generated when using this cleaning agent can have mechanical effects on material surfaces. Microorganisms can therefore penetrate prosthetic restorations more easily due to wear. In a study the effect of tea tree oil on the surface roughness of PMMA, the surface roughness value of essential oil was found to be less. This suggests that *T. vulgaris* essential oil may make the surface of denture base materials less rough. [41]

Conclusion

In this study, where the antifungal activity of TVEO was evaluated against the retention of *C. albicans* on the surface of PMMA resin material compared to Corega, TVEO showed better antifungal activity than Corega. The GC-MS analysis of the essential oil revealed the presence of volatile components including carvone, linalool, thymol, and p-cymene in significant amounts, suggesting that the antifungal activity may be attributed to these chemicals. Today, as the search for alternatives to chemical detergents continues unabated, the antimicrobial activities of various combinations of essential oils against multi-resistant microorganisms provide valuable information to the existing literature.

Ethics Committee Approval

There was no data obtained from animal or human experiments for this article.

Informed Consent

The consents were obtained from all of the authors for this article.

Author Contributions

All of the authors contributed at every stage of the study.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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