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AN ENDEMIC SPECIES *NEPETA PHYLLOCHLAMYS* P. H. DAVIS: COMPOSITION OF ESSENTIAL OIL AND ANTIMICROBIAL ACTIVITY OF EXTRACTS

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Abstract

This study investigates the composition of essential oil and biological activities of *Nepeta phyllochlamys* P. H. Davis, a local endemic species from the *Lamiaceae* family. Samples of *N. phyllochlamys* were collected from three populations, and essential oil components were compared among these populations. The main components identified in samples from three localities included caryophyllene oxide, β-pinene, linalool, T-cadinol, α-pinene, and 14-nor-kadin-5-en-4-one (isomer A). Remarkably, the chemical content of the plant exhibited variations based on geographical regions. Although the plant showed limited antibacterial and anticandidal activities in tests, this research holds significant importance due to several factors. *N. phyllochlamys* is a local endemic species and part of the widely used *Nepeta* genus in traditional practices. Moreover, there is a scarcity of studies conducted on *N. phyllochlamys*, and to the best of our knowledge, no previous research has explored its phytochemical profile and antimicrobial properties. This article contributes valuable insights into the antimicrobial effects of *N. phyllochlamys*, shedding light on its chemical composition through the application of the GC-FID and GC-MS method.

Key words: *Nepeta phyllochlamys*, antimicrobial activity, essential oil, GC-FID, GC-MS

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Introduction. There are more than 12 000 plant taxa currently growing naturally in Türkiye, and approximately 1/3 of them are endemic species. Half of the species within the *Lamiaceae* family, which comprises around 300 species, are endemic to Türkiye [1]. The *Lamiaceae* encompasses herbs, both annuals and perennials, predominantly found in the wild across Central and Southern Europe, Northern Africa, and Central and Southern Asia [2]. The genus *Nepeta*, belonging to the *Lamiaceae* family, encompasses 386 taxa (323 accepted species) worldwide according to the latest records [3]. In Türkiye, according to the Flora of Turkey [4], there are 33 *Nepeta* species, but with the recent additions of new species (*N. trautvetteri, N. janhinostegia, N. glomerata, N. roopiana, N. argolica, N. humilis*), the number of species has increased to 39. Among them, 19 species are endemic to Türkiye [1]. The genus *Nepeta* is distributed widely across Europe, North and Central America, China, India, North Africa, Southwest Asia, and Saudi Arabia. *Nepeta* species possesses a diverse chemical composition, particularly rich in terpenoids and flavonoids. Among the chemical constituents are compounds such as nepetalactones, diterpenes, triterpenes, sesquiterpenes, and iridoids [5, 6]. Several *Nepeta* species are known to attract cats, which is attributed to nepetalactone and its isomers [7]. The pharmacological and biological effects are mainly associated with nepetalactones, especially those found in *Nepeta* species.

Nepetalactones are iridoid monoterpenoids that exist in various isomeric forms in nature. *Trans*- and *cis*-isomers are highly toxic to certain insects, and many *Nepeta* species are recognized for their cat-attracting and mosquito-repellent properties. The major compounds isolated from *Nepeta* species grown in Türkiye include 4aα,7α,7aβ-nepetalactone, 4aα,7α,7aα-nepetalactone, caryophyllene oxide, 1,8-cineol, and linalool [7, 8]. *Nepeta* species are generally used as bronchodilators, diuretics, and spasmolytics. They are also widely used to alleviate various ailments, including stomach issues, eye complaints, respiratory ailments, asthma, cold, and cough. Species of the genus *Nepeta* exhibit numerous biological and pharmacological activities, such as anti-inflammatory, antioxidant, anti-Alzheimer's, anti-nociceptive, anticancer, cytotoxic, immunomodulatory, antifungal, antimicrobial, and insecticidal effects [6]. The present study focused on *N. phyllochlamys*, a local endemic species. *N. phyllochlamys*, popularly known as 'kaya pisik otu', is a Mediterranean element that grows exclusively in and around the Antalya province. There is a limited number of studies conducted on *N. phyllochlamys* species. Therefore, this study is of significant importance in terms of elucidating the volatile oil components of the plant and assessing the antimicrobial activities of the extracts obtained from it.

Materials and methods. ***Plant samples.*** The species *N. phyllochlamys* was collected from three different locations (Kemer-Antalya: L1, Kumluca-Antalya: L2, Datça-Muğla: L3). The identification was carried out using the reference "Flora of Turkey and the East Aegean Islands" [4] by Prof. Dr. Yavuz Bülent Köse. The collected plant specimens were transformed into herbarium samples and are

currently stored at the Anadolu University Faculty of Pharmacy Herbarium (ESSE 15855, 15856, 15857).

Isolation of essential oil. The aerial parts of the dried plant material were collected for the extraction of essential oil. Sixty grams of the prepared material were weighed and placed in a round-bottom flask, to which ten times its volume of distilled water was added. Subsequently, the material underwent water distillation for 3 h using a Clevenger apparatus. Due to the very low quantity of essential oil, it was taken by 1 mL *n*-hexane (Merck). The essential oil was then transferred to an amber glass vial and stored in a refrigerator at +4 °C.

GC-FID and GC-MS analysis. GC analyses were performed using an Agilent 6890N GC system. FID temperature was set to 300 °C and the same operational conditions were applied to a triplicate of the same column used in GC/MS analyses. Simultaneous auto-injection was employed to obtain equivalent retention times. Relative percentages of the separated compounds were calculated from integration of the peak areas in the GC-FID chromatograms. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system (Agilent, USA; SEM Ltd., Istanbul, Türkiye). Innowax FSC column (60 m × 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min.). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. Split ratio was adjusted to 40:1. The injector temperature was 250 °C. The interphase temperature was 280 °C. MS were taken at 70 eV. Mass range was from *m/z* 35 to 450.

Identification of compounds. The components of essential oils were identified by comparison of their mass spectra with those in the in-house Başer Library of Essential Oil Constituents, Adams Library [9], Mass Finder Library [10], Wiley GC/MS Library [11] and confirmed by comparison of their retention indices. These identifications were accomplished by comparison of retention times with authentic samples or by comparison of their relative retention index (RRI) to a series of *n*-alkanes. Alkanes were used as reference points in the calculation of relative retention indices (RRI) [12]. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Extraction. Three extracts were prepared from the aerial parts of *N. phyllochlamys* using *n*-hexane, ethyl acetate, and 70% ethanol. Eighteen grams of plant material were weighed out, and 200 mL of solvent was added. The samples were then subjected to maceration at room temperature with continuous shaking at 150 rpm using an orbital shaker for 48 h. After maceration, the extracts were filtered through filter paper, and the solvents were removed under low pressure using a rotavapor. The resulting 70% ethyl alcohol extract was stored in a lyophilizer at −20 °C for freezing. The purified extracts were kept in a refrigerator at +4 °C until they were ready for use.

Examination of antimicrobial activity. Standards and extracts were dis-

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Essential oil components of three localities

RRI	Compounds	L1 %	L2 %	L3 %	IM
1032	α-Pinene	3.1	1.5	6.8	t_R, MS
1035	α-Thujene	0.9	0.8	0.4	MS
1076	Camphene	0.1	0.2	0.1	t_R, MS
1118	β-Pinene	15.6	23.3	3.6	t_R, MS
1132	Sabinene	4.0	4.9	3.5	t_R, MS
1138	Thuja-2,4 (10)-dien	–	–	0.2	MS
1174	Myrcene	0.2	0.3	0.3	t_R, MS
1188	α-Terpinene	0.1	0.2	0.2	t_R, MS
1203	Limonene	–	0.4	0.5	t_R, MS
1213	1,8-Cineole	6.4	3.6	2.4	t_R, MS
1246	(Z)-β-Ocimene	0.3	0.6	0.2	t_R, MS
1255	γ-Terpinene	0.3	0.3	0.5	t_R, MS
1266	(E)-β-Ocimene	1.4	2.4	0.9	t_R, MS
1267	3-Octanone	0.1	0.1	0.2	t_R, MS
1280	p-Cymene	1.5	0.9	1.3	t_R, MS
1290	Terpinolene	0.1	0.1	0.1	t_R, MS
1409	Rosefuran	–	0.1	tr	MS
1450	*trans*-Linalool oxide	tr	0.3	0.7	MS
1452	1-Octen-3-ol	tr	0.1	–	t_R, MS
1452	p-Cymenene	–	–	tr	MS
1457	β-Thujone	–	0.4	tr	MS
1465	Eucarvone	–	–	tr	MS
1474	*trans*-Sabinene hydrate	–	0.7	0.3	t_R, MS
1478	*cis*-Linalool oxide	tr	0.3	0.4	MS
1523	Chrysanthenone	tr	–	–	MS
1535	Pinocamphone	–	–	0.4	MS
1544	α-Gurjunene	tr	–	–	MS
1553	Linalool	11.0	7.4	4.1	t_R, MS
1556	*cis*-Sabinene hydrate	–	0.3	0.2	t_R, MS
1571	*cis*-Dihydro-α-terpineol	–	0.1	–	MS
1571	*trans*-p-Menth-2-en-1-ol	–	–	tr	MS
1586	Pinocarvone	1.3	2.9	0.8	MS
1601	Nopinone	1.0	1.3	0.4	MS
1611	Terpinen-4-ol	–	1.2	1.1	t_R, MS
1612	β-Caryophyllene	10.0	4.4	tr	t_R, MS
1641	Thuj-3-en-10-al	1.6	–	–	MS
1648	Myrtenal	1.4	3.7	0.9	MS
1651	Sabinaketone	–	0.2	0.3	MS
1668	*cis*-Verbenol	–	–	0.7	MS
1670	*trans*-Pinocarveol	5.2	4.7	1.6	t_R, MS
1686	Lavandulol	1.6	0.6	0.6	t_R, MS
1690	*trans*-Verbenol	2.6	1.1	3.9	MS
1706	α-Terpineol	1.1	0.7	0.4	t_R, MS
1726	Verbenone	0.3	0.1	0.5	t_R, MS

T a b l e 1

Continued

1726	Germacrene D	–	0.3	tr	MS
1747	*p*-Mentha-1,5-dien-8-ol	–	–	0.8	MS
1755	Bicyclogermacrene	tr	0.1	0.3	t_R, MS
1773	δ-Cadinene	–	–	0.2	t_R, MS
1776	γ-Cadinene	–	–	5.0	MS
1802	Cumin aldehyde	–	0.3	0.2	t_R, MS
1804	Myrtenol	2.0	3.0	0.8	MS
1807	α-Cadinene	–	–	tr	MS
1845	*trans*-Carveol	–	0.2	1.0	t_R, MS
1871	*trans*-Calamenene	–	–	0.5	MS
1864	*p*-Cymen-8-ol	–	0.2	–	t_R, MS
1941	α-Calacorene	–	–	tr	MS
1953	Palustrol	tr	–	–	MS
2001	Isocaryophyllene oxide	–	1.0	–	MS
2008	Caryophyllene oxide	18.2	10.5	2.4	t_R, MS
2050	(*E*)-Nerolidol	0.8	0.6	0.3	t_R, MS
2057	Ledol	0.6	0.5	2.6	MS
2058	*p*-Mentha-1,4-dien-7-ol	–	0.4	0.2	MS
2069	Germacrene D-4β−ol	–	–	0.5	MS
2071	Humulene epoxide-II	0.7	0.5	–	MS
2080	Cubenol	–	–	1.8	MS
2089	6-Methyl-5 (3-methyl phenyl)-2-heptanone	–	–	1.6	MS
2096	Elemol	2.2	3.1	–	MS
2113	Cumin alcohol	tr	0.3	–	t_R, MS
2144	Spathulenol	0.9	1.0	2.2	t_R, MS
2185	γ-Eudesmol	tr	1.0	–	MS
2187	T-Cadinol	–	–	14.9	MS
2250	α-Eudesmol	0.4	0.4	–	MS
2255	Cadalene	–	–	1.6	MS
2257	β-Eudesmol	0.6	0.5	–	MS
2264	4,7-Dimethyl-1-tetralone	–	–	0.9	MS
2320	14-Nor-cadin-5-en-4-one (isomer A)	–	–	6.8	MS
2324	Caryophylladienol II	tr	0.7	–	MS
2349	Cadina-4, 10 (15)-dien-3-one	–	–	5.6	MS
2389	Caryophyllenol I	tr	–	–	MS
2392	Caryophyllenol II	1.3	–	–	MS
2411	4-Isopropyl-6-methyl-1-tetralone	–	–	0.7	MS
	Total %	98.9	98.4	89.4	

RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from the FID chromatograms; tr: Trace (<0.1%). Identification method (IM): t_R, identification based on the retention times of genuine compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the in-house Başer Library of Essential Oil Constituents, Adams, Mass Finder and Wiley libraries and comparison with literature data

solved in sterile DMSO (Dimethyl sulfoxide, Carlo Erba®) and used. Ampicillin (Sigma aldrich®) and Chloramphenicol (Sigma aldrich®) were used as positive controls in antibacterial tests, and Amphotericin-B (Sigma aldrich®) and Ketoconazole (Thermo scientific®) were used in anticandidal tests.

Antibacterial activity test. The minimum inhibitory and bactericidal (MIC and MBC) concentrations of the extracts were determined using standard protocols (CLSI M7-A7). The strains used for testing were *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* NRRL B-2544 and *Klebsiella pneumoniae* NCTC 9633 [13].

Anticandidal activity test. MIC concentrations of the extracts were determined using standard protocols (CLSI M27-A2). The strains used for testing were *Candida utilis* NRRL Y-900, *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 [14].

Results. ***Essential oil components.*** As a result of hydrodistillation of the aerial parts a pungent, slightly yellow coloured essential oil was obtained. The components of the essential oil were determined by gas chromatography system (GC-FID and GC-MS) and were shown in Table 1. In the data of Antalya-Kemer, the main components were determined as caryophyllene oxide (18.2%), β-pinene (15.6%) and linalool (11.0%), respectively. While in the Antalya-Kumluca locality, the main components were β-pinene (23.3%), caryophyllene oxide (10.5%) and linalool (7.4%); in the Muğla-Datça locality, T-cadinol (14.9%), α-pinene (6.8%), 14-nor-kadin-5-en-4-one (isomer A) (6.8%) (Table 1).

Antibacterial activity results. The extracts were tested against 4 bacterial species. The extracts were diluted in the range of 62.5–8000 µg/mL. Inhibition effects were observed in the range of 2000–8000 µg/mL as MIC. The ethanol extract did not show any inhibitory effect against *Staphylococcus aureus* and *Serratia marcescens* at the highest test dose. The minimum bactericidal dose for all extracts was bigger than the higher concentration, 8000 µg/mL (Table 2).

T a b l e 2

Antibacterial effect results of the extracts (µg/mL)

Bacteria	N-H	N-EtOAc	N-EtOH	CLR	AMP
Staphylococcus aureus ATCC 6538	2000	2000	>8000	4	1
Pseudomonas aeruginosa ATCC 27853	4000	2000	4000	8	>64
Serratia marcescens NRRL B-2544	4000	4000	>8000	4	>64
Klebsiella pneumoniae NCTC 9633	4000	4000	8000	2	>16

N-H: *N. phyllochlamys n*-hexane extract, N-EtOAc: Ethyl acetate, N-EtOH: 70% ethanol, CLR: Chloramphenicol, AMP: Ampicillin-Na.

Anticandidal activity. The extracts were tested against five reference strains of *Candida*. It was found that the *n*-hexane extract exhibited the highest anticandidal activity having a MIC value of 125 µg/mL against *C. tropicalis* (Table 3).

T a b l e 3

Anticandidal effect results of extracts (µg/mL)

Candida	N-H	N-EtOAc	N-EtOH	AMP-B	Kt
C. utilis NRRL Y-900	500	500	1000	1	0.5
C. albicans ATCC 90028	2000	2000	4000	1	0.5
C. tropicalis ATCC 750	125	500	250	2	0.25
C. parapsilosis ATCC 22019	2000	2000	4000	2	0.125
C. krusei ATCC 6258	4000	4000	4000	1	1

N-H: *N. phyllochlamys* *n*-hexane, N-EtOAc: Ethyl acetate, N-EtOH: 70% ethanol, AMP-B: Amphotericin B, Kt: Ketoconazole

Discussion. ***Essential oil composition.*** These variations in chemical composition can be attributed to the differences in location, aspect, and altitude, which affect the plant's adaptation to its environment. *Nepeta* species generally contains nepetalactone derivatives as the main component (4aβ,7α,7aα-nepetalactone; 4aα,7α,7aα-nepetalactone; 4aα,7β,7aα-nepetalactone; 4aα,7β,7aβ-nepetalactone; 4aβ,7α,7aβ-nepetalactone). Furthermore, other compounds such as 1,8-cineol, α or β-caryophyllene, β-caryophyllene oxide, α-pinene, and β-pinene have been found abundantly in this genus [15].

In this study, it was observed that the main components of the species collected from different localities consisted of components such as caryophyllene oxide, β-pinene and linalool, T-cadinol, α-pinene and 14-nor-kadin-5-en-4-one (isomer A). The results were similar to the compounds found extensively in *Nepeta* species, but nepetalactones were not found in this species. Sesquiterpene hydrocarbons (19.8–31.4%), monoterpene hydrocarbons (21.8–34.5%) and oxygenated monoterpenes (4.8–10%) were the major terpene groups in the oil of *N. phyllochlamys*. Only one previous work has reported the composition of essential oil of *N. phyllochlamys*. The main components of the plant were found to be β-pinene (16.26%), terpinene-4-ol and β-caryophyllene (8.37%), caryophyllene oxide (7.78%), linalool (7.64%), sabinene (6.56%), and 1,8-cineole (5.91%), respectively. The results of this study were found to be quite similar to our results [7]. But as a result of the plant re-identification made by Prof. Yavuz Bülent Köse, it was understood that the species was *Nepeta nuda.* Hence, this study can be regarded as the first essential oil composition investigation specifically conducted on *N. phyllochlamys*.

Antibacterial activity. In a previous study reported by Nadeem et al. [16], several solvent extracts of *N. cataria* aerial parts were evaluated for their

antibacterial activity against 5 gram-negative and 5 gram-positive bacteria using disc diffusion and microdilution methods. The ethanol, methanol, and water extracts exhibited inhibition against all tested bacteria at concentrations ranging from 250 to 1000 µg/mL. Acetone and hexane extracts showed weak inhibition against the tested bacteria when compared to standard antimicrobials. In another study, the in vitro antimicrobial activities of the essential oil and methanol extract of *N. cataria* were investigated. The results showed that the methanol extract of *N. cataria* demonstrated remarkable antimicrobial activity against *Staphylococcus aureus* A215 at the concentration of 62.5 µg/mL (MIC). The activity of the methanol extract from *N. cataria* was found to be less potent when contrasted with the essential oil. The methanol extract of *N. cataria* exhibited weaker activity compared to the essential oil [17]. In this study, the extracts of *N. phyllochlamys* demonstrated inhibitory effects between the concentrations of 2000–8000 µg/mL. To the best of our knowledge, the present study is the first antimicrobial work on *N. phyllochlamys.*

Anticandidal activity. In the study of ADIGÜZEL et al. [17], the antimicrobial activity of *N. cataria* was investigated. The essential oil demonstrated inhibition effect against eleven bacteria, twelve fungi and one yeast (*C. albicans*), with MIC values ranging from 12.50 to 250 µl/mL. But the methanol extract showed lower inhibitory effect. İŞCAN et al. [18] demonstrated that methanol extract of *N. cilicica* exhibited potent inhibition against *S. aureus* and *C. tropicalis* (47 µg/mL MIC). The ethanolic extract of *N. trachonitica* has demonstrated a strong antibacterial effect against *E. coli* (inhibition zone: 12.0 ± 1.24). However, the extracted sample exhibited a comparatively mild impact on *P. aeruginosa* and displayed negligible activity against *K. pneumoniae. N. trachonitica* also exhibited notable antifungal properties. The ethanolic extract displayed its highest susceptibility against *S. cereviciae*, followed closely by *C. albicans.* Inhibition zones for these two fungal strains measured 19.0 ± 1.69 and 13.0, respectively [19]. MIC values against *C. albicans* were found to be 2000 µl/mL for *n*-hexane and ethyl acetate, and 4000 µl/mL for ethanol extract. In this study was found that the *n*-hexane extract exhibited the highest anticandidal activity having a MIC value of 125 µg/mL against *C. tropicalis.* The extracts of *N. phyllochlamys* exhibited limited inhibitory effects against the tested *Candida* strains when compared to standard anticandidal agents.

To the best of our knowledge, no previous study of anticandidal activity has been reported for *N. phyllochlamys.* Therefore, this study represents the first investigation of the anticandidal effects of *N. phyllochlamys*, and it is important in terms of contributing new experimental results to the existing literature. In conclusion, our study adds to the growing body of knowledge regarding the antimicrobial properties of *Nepeta* extracts. The research underscores the diversity of antimicrobial effects among different *Nepeta* species, emphasizing the importance of further exploration to harness their therapeutic potential effectively.

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