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Composition, and antioxidant and antimicrobial activities of the essential oils of a full-grown *Pinus cembra* L. tree from the Calimani Mountains (Romania)

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Abstract: The chemical composition and the antioxidant and antimicrobial effects of the essential oils of *Pinus cembra* L. needles and twigs were investigated in this study. The chemical composition was analyzed using both the GC and GC–MS techniques. α-Pinene (69.14 %) was the major constituent of the needle essential oil while the twig essential oil was characterized by a high content of limonene+β-phellandrene (40.97 %) and α-pinene (24.94 %). The needle and twig essential oils showed weak DPPH radical scavenging effects. In the antimicrobial assays, both essential oils showed high activity against *Sarcina lutea* and *Staphylococcus aureus* but no activity against *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The needle and twig essential oils had similar antimicrobial effects against *Sarcina lutea*. The twig essential oil was more active against *S. aureus* than the needle essential oil and also exhibited a moderate activity against *Candida albicans*.

Keywords: cembran pine; α-pinene; limonene; DPPH radical; *Staphylococcus aureus*; *Candida albicans*.

INTRODUCTION

Pinus cembra L. (Pinaceae), commonly known as cembran pine, Swiss stone pine or Arolla pine, is a glacial relict growing in the European Alps (Switzerland, Austria, northern Italy and south-eastern France) and the Carpathian Mountains

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(Poland, Slovakia, Ukraine and Romania). It grows at high altitudes ranging from 900 to 2850 and 1985 m in the Alps and the Carpathian Mountains, respectively, and is very resistant to the conditions of high abiotic stress in the alpine timberline (UV-B irradiation, frosts and strong winds).[1–3]

The chemistry and the biological effects of essential oils of different pine species have been intensively studied.[4–9] In contrast, there are only a few studies on cembran pine essential oils.[10,11] Dormont *et al.* studied the volatiles emitted by the cones and foliage (ramets with one-year-old needles from the apical parts of branches) and reported α- and β-pinene together with a mixture of limonene and β-phellandrene as the major volatile constituents; other monoterpene hydrocarbons (myrcene, camphene, sabinene, terpinolene and bornyl acetate) were identified in traces. In addition, traces of tricyclene were found in the cone volatiles.[10] Ochocka *et al*. separated, identified and quantified the enantiomers of four monoterpene hydrocarbons (α- and β-pinene, camphene and limonene) in the needle essential oil. The authors reported the occurrence of both enantiomers of camphene, α- and β-pinene and the (–)isomer of limonene. (–)Limonene and α-pinene as sum of the (–) and (+)isomers dominated in the needle essential oil.[11] A literature survey revealed no studies on the biological effects of cembran pine volatiles. In this context, the main objectives of the present study were to investigate the chemical composition of the needle and twig essential oils of *P. cembra* and to evaluate their antioxidant and antimicrobial effects in order to find some possible therapeutic uses.

EXPERIMENTAL

Plant material

The needles with twigs were sampled from a full-grown cembran pine in a natural stand located at approximately 1650 m altitude on the Calimani Mountains (Romania) in June 2011. The plant material was authenticated in the Department of Plant and Animal Biology, School of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" – Iasi, Romania. Before extraction of the essential oils, the needles and twigs were separated and dried in the shade at 20±2 °C. Herbarium voucher samples have been deposited in the Department of Pharmacognosy, School of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" – Iasi, Romania.

Chemicals

An alkane standard solution C_8–C_{20}, butylated hydroxyanisole (BHA) and 2,2-diphenyl--1-picrylhydrazyl (DPPH) radical were purchased from Sigma-Aldrich (Steinheim, Germany). Mueller Hinton agar and broth were obtained from Merck (Darmstadt, Germany). Sabouraud 4 % glucose agar was obtained from Fluka Biochemika (Buchs, Switzerland). Sabouraud dextrose broth was procured from Oxoid (Basingstoke, UK). The antibiotic discs were purchased from Himedia (Mumbai, India). All other reagents and solvents were of analytical grade.

Microorganisms

Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341 and *Bacillus cereus* ATCC 14579), Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and pathogenic yeasts (*Candida albicans* ATCC 10231) were obtained from the Culture Collection of the Department of Microbiology, School of Medicine, University of Medicine and Pharmacy "Grigore T. Popa" – Iasi, Romania.

Isolation of the essential oils

100 g of each sample (needles or twigs) were ground with a grinder, mixed with 1000 mL of doubly distilled water and subjected to hydrodistillation for 4 h in a modified Clevenger-type apparatus.[9] The essential oils were collected, dried over anhydrous sodium sulphate and kept in a sealed glass tube at 4 °C until studied. Yields of the essential oils (v/w %) were calculated on a dry weight basis and are expressed as average values of the results from several extractions.

GC and GC–MS analyses

The GC-FID analysis of the essential oils was performed on an Agilent 6890 gas chromatograph. Separation of the constituents of the essential oils was realised on a DB-5MS capillary column (30 m×0.25 mm i.d., film thickness 0.25 μm). A volume of 0.3 μL was injected in the split mode (split ratio 1:100). The carrier gas was helium at a flow rate of 1.0 mL min^{-1}. The oven temperature was programmed from 40 to 280 °C at a rate of 3 °C min^{-1}; the final temperature was held for 8 min.[7] The injector and detector temperatures were maintained at 250 °C. The GC–MS analysis was performed using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C inert mass selective detector with electron impact ionization. The same column and temperature program as described for GC-FID analysis were used. The carrier gas was helium (1.0 mL min^{-1}) and the split ratio was 1:100. The injector, MS source and MS quadrupole temperatures were set at 250, 230 and 150 °C, respectively. Mass spectra were acquired in the scan mode (mass range 15–450 *m*/*z*). In order to determine the retention indices, standard solutions of *n*-alkanes (C_8–C_{20}) were analyzed under the same chromatographic conditions. The volatile constituents were identified by comparison of their mass spectral data with those in the Wiley 275 mass spectra library stored in the GC–MS database and with those of pure standards. The identity of the constituents was confirmed by comparison of calculated retention indices with those reported in the literature[12-16] and with those of pure standards. The relative percentages of the essential oils constituents were obtained from the FID peak areas without the use of correction factors.

DPPH radical scavenging assay

DPPH radical scavenging activity was evaluated as described by Mighri *et al.*[17] Briefly, 1 mL of each essential oil dilution in methanol (concentration range from 20 to 60 mg mL^{-1}) was mixed with 1 mL DPPH solution in methanol (0.004 %, w/v). The mixtures were vortexed and kept in the dark. After 30 min, the absorbance was measured at 517 nm. BHA was used as a positive control. DPPH radical scavenging activity (%) was calculated using the following formula:

$$100(A_0 - A_t)/A_0$$

where A_0 is the absorbance of the blank and A_t is the absorbance in the presence of an essential oil or positive control. All the experiments were performed in triplicate; the results are expressed as means ± *SD*. The EC_{50} (the concentration that gives half-maximal response) values were calculated by linear interpolation between the values above and below 50 % activity.

Agar diffusion assay

The antimicrobial activities were screened using the agar diffusion assay.[18,19] Each microbial suspension was adjusted to a turbidity equivalent to 0.5 McFarland turbidity standard. Aliquots (0.1 mL) of each microbial suspension were spread in Mueller Hinton agar (antibacterial tests) and Sabouraud agar (antifungal tests). Sterile stainless steel cylinders (5 mm internal diameter; 10 mm height) were placed on the agar surface and filled with 30 µL of each essential oil. Discs (6 mm diameter) containing ampicillin (25 µg disc^{-1}), chloramphenicol (30 µg disc^{-1}) and nystatin (100 µg disc^{-1}) were used as positive controls. The plates were incubated at 37 °C for 24 h (antibacterial tests) and at 24 °C for 48 h (antifungal tests). After incubation, the diameters of the inhibition zones were measured. All experiments were performed in triplicate and the results are expressed as means ± *SD*.

Broth microdilution assay

The minimum inhibitory concentrations (*MIC*s) and the minimum bactericidal/fungicidal concentrations (*MBC*s/*MFC*s) were determined using a broth microdilution assay.[17,20] The microbial suspensions were adjusted to a turbidity of 0.5 McFarland standard. The essential oils were dissolved in 50 % dimethyl sulphoxide at a concentration of 250 mg mL^{-1} and then subjected to serial two-fold dilutions in Mueller Hinton broth (antibacterial tests) and Sabouraud broth (antifungal tests) in 96-well plates (concentration range from 250 to 0.12 mg mL^{-1}; final volume in each well 100 µL). Other 95 µL of broth and 5 µL of microbial inoculum were further dispensed into each well. The plates were incubated at 37 °C (antibacterial tests) and 24 °C (antifungal tests) for 24 h. The essential oils were screened twice against each microorganism. The lowest concentration of the essential oil that inhibited the visible growth of microorganisms was recorded as the *MIC*.[21] The *MBC*/*MFC* values (the lowest concentration of an essential oil killing completely the microorganisms being tested)[21] were determined by transferring 10 µL of samples showing inhibition of visible growth on the surface of an agar plate. The subcultures were incubated at 37 °C (antibacterial tests) and 24 °C (antifungal tests) for 24 h. The *MIC* and *MBC*/*MFC* values of ampicillin/nystatin towards bacteria/yeast strains were also evaluated.

Statistical analysis

Statistical analyses were performed using the Kruskal–Wallis and Mann–Whitney U tests. Values of *P* less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Essential oils analysis

The essential oils from needles (EON) and twigs (EOT) of cembran pine were obtained in yields of 1.96 and 2.45 %, respectively and analyzed by both GC and GC–MS. A list of the constituents in order of elution on a DB-5MS column, their retention indices and percentages are reported in Table I. It is noteworthy that in both essential oils, limonene co-eluted with *β*-phellandrene and the two compounds were quantified as a single peak. 27 compounds accounting for 91.59 % of the total essential oil composition were identified in EON. *α*-Pinene (69.14 %) was the most abundant compound. Other main constituents were limonene+*β*-phellandrene (4.64 %), *α*-cadinene (3.71 %), *γ*-cadinene (2.61 %), camphene (2.59 %), bicyclogermacrene (2.44 %) and cadina-1,4-diene (1.36 %). The

amount of β-pinene was found to be 0.89 %. Monoterpene hydrocarbons (78.93 %) were the major class of compounds followed by sesquiterpene hydrocarbons (12.09 %). GC analysis of EOT revealed 28 compounds representing 92.22 % of the total essential oil composition. The main constituents of EOT were limonene+β-phellandrene (40.97 %), α-pinene (24.94 %) and β-pinene (10.38 %), followed by camphene (5.55 %), myrcene (1.7 %) and γ-terpinene (1.52 %). Monoterpene hydrocarbons (87.24 %) were the most abundant while sesquiterpene hydrocarbons (3.35 %) were present in lower amounts.

TABLE I. Chemical composition (%) of cembran pine essential oils

Compound	*RI*[a]	*RI*[b]	EON	EOT
2-Hexenal	850	850	0.17	–
Tricyclene	921	926	0.14	0.30
α-Thujene	925	924	–	tr[c]
α-Pinene	936	935	69.14	24.94
Camphene	950	953	2.59	5.55
Sabinene	970	975	tr	–
β-Pinene	979	979	0.89	10.38
Myrcene	990	990	0.58	1.7
α-Phellandrene	1006	1004	–	0.23
δ-3-Carene	1008	1009	0.69	1.03
p-Cymene	1023	1024	tr	–
Limonene+β-phellandrene	1028	1029	4.64	40.97
γ-Terpinene	1058	1058	–	1.52
α-Terpinolene	1084	1084	0.16	0.56
Linalool	1099	1101	tr	–
Nonanal	1105	1102	–	0.49
trans-Pinocarveol	1139	1139	–	0.10
Terpineol-4	1179	1177	–	0.10
Estragole	1197	1200	0.23	0.21
trans-Carveol	1218	1219	–	0.14
Carvone	1242	1243	–	0.23
Bornyl acetate	1283	1283	0.16	0.20
α-Cubebene	1344	1349	tr	–
α-Copaene	1372	1375	tr	–
β-Caryophyllene	1415	1415	0.96	0.34
α-Humulene	1452	1451	0.17	0.81
γ-Muurolene	1472	1475	tr	tr
Germacrene D	1478	1480	tr	–
β-Selinene	1485	1484	0.22	0.15
Bicyclogermacrene	1492	1488	2.44	–
α-Muurolene	1495	1499	0.24	0.21
γ-Cadinene	1510	1514	2.61	0.23
δ-Cadinene	1516	1523	0.31	0.38
Cadina-1,4-diene	1529	1531	1.36	0.41
α-Cadinene	1533	1537	3.71	0.73
Caryophyllene oxide	1578	1581	–	0.16

TABLE I. Continued

Compound	*RI*[a]	*RI*[b]	EON	EOT
Monoterpene hydrocarbons			78.93	87.24
Oxygenated monoterpenes			0.24	0.78
Sesquiterpene hydrocarbons			12.09	3.35
Oxygenated sesquiterpenes			–	0.16
Others			0.33	0.69
Total identified			91.59	92.22

[a]Retention indices relative to C_8–C_{20} *n*-alkanes calculated on DB-5MS capillary column; [b]retention indices reported in the literature;[12-16] [c]traces (<0.1 %)

A previous study made by Dormont *et al.* reported α-pinene, β-pinene and a mixture limonene+β-phellandrene as the major constituents in the volatiles emitted by cembran pine cones and foliage (ramets with one-year-old needles from the apical parts of the branches). α-Pinene (67.1 %) dominated in the cone volatiles while β-pinene (18.2 %) and limonene+β-phellandrene (11.1 %) were in lower amounts. Regarding foliage volatiles, the authors reported differences between samples collected from forest trees (α-pinene 38.9 %, β-pinene 20.7 %, limonene+β-phellandrene 34.0 %) and planted trees (α-pinene 26.4 %, β-pinene 10.1 %, limonene+β-phellandrene 57.9 %).[10] Ochocka *et al.* studied a commercially manufactured essential oil from cembran pine needles. This study revealed that α-pinene as the sum of enantiomers and limonene as the (–)isomer were present in similar amounts (34.3 and 32.7 %, respectively); in case of β-pinene, the sum of both enantiomers was only 12.5 %.[11] On the contrary, in the present study, α-pinene was identified in the highest percentage (69.14 %) in the needle essential oil while limonene+β-phellandrene and β-pinene were in significantly lower amounts (4.64 and 0.89 %, respectively). These differences in the chemical composition of essential oils from cembran pine needles can most probably be explained by different environmental conditions and time of collection.

Antioxidant activity

The antioxidant activities of cembran pine essential oils were evaluated by the DPPH radical scavenging assay, which is a simple, rapid and highly reproducible method widely used in antioxidant screening.[22] The results of the assay are given in Table II. According to the EC_{50} values, both EON (19.93±0.75 mg mL^{-1}) and EOT (18.66±0.70 mg mL^{-1}) were less active than the positive control, BHA (3.3±0.1 μg mL^{-1}). The weak antioxidant activity of cembran pine essential oils might be related to the high content of non-phenolic compounds (monoterpenes and sesquiterpenes). Among plant secondary metabolites, polyphenols are known to have a high capacity to scavenge free radicals due to the hydrogen and electron donating abilities of their hydroxyl groups.[22]

There are very few literature reports on DPPH scavenging effects of the essential oils from other *Pinus* species. Different experimental protocols in DPPH

assay make it difficult to compare the present results with those of other studies. However, it should be pointed out that a weak DPPH scavenging activity (less than 20 %) was reported for the essential oil from *P. radiata* (10 μL) after 70 min reaction time.[23] On the contrary, high DPPH scavenging effects were reported for the essential oils from *P. densiflora* and *P. thunbergii* needles (EC_{50} = 120 and 30 μg mL^{-1}, respectively) in comparison to *α*-tocopherol (EC_{50} = 12.6 μg mL^{-1}) and butylated hydroxytoluene (EC_{50} = 14.3 μg mL^{-1}); the scavenging effects were assessed after 30 min reaction time at 37 °C.[24] These contradictory results might be due to different experimental conditions in the DPPH assay, but also to differences in the chemical composition and possible interactions between the volatile constituents.[25]

TABLE II. DPPH Radical scavenging activity of cembran pine essential oils; different letters in the column indicate values significantly different ($P \leq 0.05$)

Essential oil/positive control	EC_{50}
EON	19.93±0.75 mg mL^{-1}[a] (b)
EOT	18.66±0.70 mg mL^{-1}[a] (b)
BHA	3.3±0.1 μg mL^{-1}[a] (a)

Antimicrobial activity

The antimicrobial activities were initially tested by the agar diffusion method in comparison to ampicillin, chloramphenicol and nystatin (Table III). EON and EOT showed high activity against *S. aureus* and *S. lutea* with inhibition zones larger than 14 mm but no activity against *B. cereus*, *E. coli* and *P. aeruginosa*. The EOT also had moderate activity against *C. albicans* (inhibition zone of 9.66 mm).[26] Unlike EOT, EON showed no antifungal activity.

TABLE III. Antimicrobial activity (diameter of inhibition zone, mm) of cembran pine essential oils determined by the agar diffusion method; different letters in the column indicate values significantly different ($P \leq 0.05$)

Microorganism	EON (30 μL)	EOT (30 μL)	Ampicillin (25 μg $disc^{-1}$)	Chloramphenicol (30 μg $disc^{-1}$)	Nystatin (100 μg $disc^{-1}$)
S. aureus ATCC 25923	22[a] (a)	24.7±0.6[a] (b)	27.7±0.6[a] (c)	26±1[a] (bc)	Not determined
S. lutea ATCC 9341	31±1[a] (a)	30±1[a] (a)	36[a] (b)	35.3±0.6[a] (b)	Not determined
B. cereus ATCC 14579	No zone	No zone	No zone	25.7±0.6	Not determined
E. coli ATCC 25922	No zone	No zone	19±1[b] (a)	26[b] (b)	Not determined
P. aeruginosa ATCC 27853	No zone	No zone	No zone	18	Not determined
C. albicans ATCC 10231	No zone	9.7±0.6[b] (a)	Not determined	Not determined	29.3±0.6[b] (b)

The *MIC* and *MBC*/*MFC* values of cembran pine essential oils against the sensitive microbial strains were determined by a broth microdilution assay in comparison to ampicillin and nystatin (Table IV). Both essential oils showed similar antimicrobial effects against *S. lutea* with *MIC* and *MBC* values of 0.12 and 0.24 mg mL^{-1}, respectively. *S. aureus* was less susceptible to cembran pine essential oils. According to the *MIC* and *MBC* values, EOT (1.95 and 3.9 mg mL^{-1}, respectively) was more active than EON (3.9 and 15.62 mg mL^{-1}, respectively). The broth microdilution assay confirmed the antifungal activity of EOT (*MIC* = 7.81 mg mL^{-1} and *MFC* = 15.62 mg mL^{-1}).

TABLE IV. Antimicrobial activity of cembran pine essential oils determined by the broth microdilution method

Microorganism	EON		EOT		Ampicillin		Nystatin	
	MIC	*MBC*/*MFC*	*MIC*	*MBC*/*MFC*	*MIC*	*MBC*/*MFC*	*MIC*	*MBC*/*MFC*
	mg mL^{-1}				μg mL^{-1}			
S. aureus ATCC 25923	3.9	15.62	1.95	3.9	0.25	0.5	n.d.[a]	n.d.
S. lutea ATCC 9341	0.12	0.24	0.12	0.24	0.25	0.5	n.d.	n.d.
C. albicans ATCC 10231	n.d.	n.d.	7.81	15.62	n.d.	n.d.	1	2

[a]Not determined

Among volatile constituents, phenolics (thymol, carvacrol and eugenol) and oxygenated monoterpenes (*α*-terpineol, terpinen-4-ol and linalool) have been reported to possess not only strong antimicrobial effects, but also a wide spectrum of activity.[27] The lack of activity against some microbial strains might be due to the high content of monoterpene hydrocarbons in the cembran pine essential oils. The low antimicrobial activity of hydrocarbons has been attributed to their low hydrogen bonding capacity and water solubility.[28] Both EON and EOT are dominated by monoterpene hydrocarbons; they contain low amounts of oxygenated monoterpenes (< 1 %) and no phenolics. EOT proved to be more effective than EON. The higher percentages of limonene+*β*-phellandrene and *β*-pinene are not solely responsible for the antimicrobial potency of EOT; synergistic, additive or antagonistic interactions between volatile constituents may also affect the antimicrobial activity.

There are many literature reports on the antimicrobial effects of other pine needle essential oils, some of them giving contradictory results. The essential oil isolated from the needles of *P. nigra* ssp. *dalmatica* was reported to have a high effect against *S. aureus* (*MIC* = 0.03 %, v/v) and less pronounced effects against *P. aeruginosa* (*MIC* = 0.25 %, v/v), *E. coli* (*MIC* = 2.50 %, v/v) and *C. albicans* (*MIC* = 0.25 %, v/v).[8] Hong *et al*. reported that the essential oil from *P. koraiensis* needles showed no activity against *S. aureus* and *E. coli* but exhibited

antifungal activity against *C. albicans* (28.9–31.5 % *vs*. control); the essential oil from *P. densiflora* needles inhibited the growth of *S. aureus* (66.7–77.8 % *vs*. control) while it had no activity against *E. coli* and *C. albicans*.[29] On the contrary, Park and Lee reported that the essential oil from *P. densiflora* needles exhibited antibacterial effects against *E. coli*, *S. aureus* and *P. aeruginosa* with *MIC* values of 0.8, 1.7 and 26.3 mg mL^{-1}, respectively. Against the same bacterial strains, the *MIC* values of the essential oil from *P. thunbergii* needles were 6.6, 13.2 and 26.3 mg mL^{-1}, respectively.[24] These differences in antimicrobial effects of pine essential oils might be attributed to different antimicrobial screening assays, variations in chemical composition and possible interactions between the constituents.[30]

CONCLUSIONS

This is the first report on the antioxidant and antimicrobial effects of the essential oils of cembran pine needles and twigs and also the first report on the chemical composition of the latter. The results of the present study indicate that both essential oils are rich in monoterpene hydrocarbons. α-Pinene was the major constituent of the needle essential oil while the twig essential oil was dominated by limonene+β-phellandrene and α-pinene. Cembran pine essential oils showed weak free radical scavenging activity but displayed high antibacterial activity against *S. lutea* and *S. aureus*. Twig essential oil was more active against *S. aureus* than needle essential oil and, in addition, it was active against *C. albicans*. Further research is required to evaluate the spectrum of activity of cembran pine essential oils and the possible interactions (synergistic, additive and antagonistic) in the case of association with antibiotics and other essential oils.

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ИЗВОД

САСТАВ, АНТИОКСИДАТИВНА И АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКИХ УЉА ДРВЕТА *Pinus cembra* СА ПЛАНИНА КАЛИМАНИ (РУМУНИЈА)

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Испитиван је хемијски састав, антиоксидативна и антимикробна активност етарских уља из иглица и грана дрвета *Pinus cembra* L. Хемијски састав је утврђиван мето-

дама GC и GC–MS. α-Пинен је био главни састојак етарског уља из иглица (69,14 %), док је етарско уље грана највише имало лимонена и β-феландрена (укупно 40,97 %) и α-пинена (24,94 %). Етарска уља иглица и грана су исказала малу активност у DPPH тесту. Оба уља су испољила велику активност према бактеријама *Sarcina lutea* и *Staphylococcus aureus*, али не према *Bacillus cereus*, *Escherichia coli* и *Pseudomonas aeruginosa*. Етарска уља иглица и грана су имала сличну антимикробну активност према *S. lutea*. Етарско уље из грана је било активније према *S. aureus* него уље из иглица, а испољавало је извесну активност и према гљивици *Candida albicans*.

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