

Antimikotsko djelovanje tekućeg ekstrakta i eteričnog ulja iz plodova aniša (*Pimpinella anisum* L., Apiaceae)

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Source / Izvornik: **Acta Pharmaceutica, 2005, 55, 377 - 385**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

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Download date / Datum preuzimanja: **2023-11-30**



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Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., *Apiaceae*)

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Antifungal activities of fluid extract and essential oil obtained from anise fruits *Pimpinella anisum* L. (*Apiaceae*) were tested *in vitro* on clinical isolates of seven species of yeasts and four species of dermatophytes. Diffusion method with cylinders and the broth dilution method were used for antifungal activity testing. Anise fluid extract showed antimycotic activity against *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis* and *C. krusei* with MIC values between 17 and 20% (V/V). No activity was noticed against *C. glabrata*, and anise fruits extracts showed growth promotion activity on *Geotrichum* spp. Anise fruits extract inhibited the growth of dermatophyte species (*Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis* and *M. gypseum*) with MIC values between 1.5 and 9.0% (V/V). Anise essential oil showed strong antifungal activity against yeasts with MIC lower than 1.56% (V/V) and dermatophytes with MIC lower than 0.78% (V/V). Significant differences in antifungal activities were found between anise fluid extract and anise essential oil ($p < 0.01$). Anise essential oil exhibited stronger antifungal activities against yeasts and dermatophytes with MIC values between 0.10 and 1.56% (V/V), respectively.

Keywords: anise, anise fruit, *Pimpinella anisum* (*Apiaceae*), fluid extract, essential oil, antifungal activity, *Candida*, dermatophyte

Received December 4, 2004
Accepted July 5, 2005

Plants are a large source of new bioactive molecules with therapeutic potentials. Only a small percentage of living plants on Earth have been phytochemically investigated. Plants are thus an enormous reservoir of pharmaceutically valuable molecules to be discovered (1, 2).

Although there are drugs effective against yeasts and dermatophytes, plant extracts as well as essential oils and other compounds are of considerable interest because of their antifungal activity (3–5). In the last ten years there has been a noticeable increase of mycoses, localized on the skin or mucous and systematic caused by yeast species from

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the genus *Candida* (6, 7), as well as by moulds from the genera *Aspergillus*, *Fusarium* and *Zygomycetes* (8), especially in the group of immunocompromised patients. Antifungal therapy is often limited by the resistance of yeasts to used antimycotics (7).

Because of the mentioned facts, searching for sources of new antimycotics is justified, with plants providing a promising source of new substances with antifungal activity.

Anise (*Pimpinella anisum* L., *Apiaceae*) is an annual herb indigenous to Near East and widely cultivated in the Mediterranean rim (Turkey, Egypt, Syria, Spain, etc.) and in Mexico and Chile. It has been used as an aromatic herb and spice since Egyptian times and antiquity and has been cultivated throughout Europe (9). In folk medicine, anise is used as an appetizer, tranquillizer and diuretic drug (10, 11). The traditional use of Pernod, Ouzo, Anisette, Raki, and many other anise-flavoured drinks after a heavy meal is a familiar example of its antispasmodic effect, especially in the digestive tract (9). Dried ripe fruits of anise, commercially called aniseeds (Anisi fructus), contain the whole dry cremocarp of anise (*P. anisum* L.). For medical purposes, they are used to treat dyspeptic complaints and catarrh of the respiratory tract, and as mild expectorants. It was also reported that extracts from anise fruits have therapeutic effects on several conditions, such as gynaecological and neurological disorders (11, 12). Ethanolic extract of anise-fruits contains *trans*-anethole, methylchavicol (estragole), eugenol, psideoisoeugenol, anisaldehyde, coumarins (umbelliferon, scopoletin), caffeic acid derivatives (chlorogenic acid), flavonoids, fatty oil, proteins, minerals, polyenes and polyacetylenes as its major compounds (9).

Anise oil (Anisi aetheroleum) is the essential oil obtained by steam distillation from dry ripe fruits of anise (*P. anisum* L.) or star anise (*Illicium verum* Hook. fil.) (13). Essential oil of anise fruits contains from 80 to 95%, or more, *trans*-anethole as the main compound, followed by chavicol methyl ether (estragole), anisaldehyde and *cis*-anethole (9, 10). Oil is used as an expectorant and carminative, especially in pediatrics, in cough mixtures and as a flavouring and spice. The important phenylpropanes, such as *trans*-anethole and chavicol methyl ether (estragole), have a stabilizing influence on the autonomous nervous system. Anise oil has been used in Iranian folk medicine for the treatment of some diseases, including seizures and epilepsy (14). Anise has mild estrogenic effects, which explains the use of this plant in folk medicine for increasing milk secretion (12) and for amenorrhea. Products that contain anise fruits extracts or anise essential oil may cause contact dermatitis, probably due to their anethole content (15).

Because of the interesting biological effects of anise fruits, we have decided to examine and compare the *in vitro* antifungal effects of the liquid extract and the essential oil of anise fruits against the most common clinical isolates of yeasts and against dermatophytes.

EXPERIMENTAL

Reagents and solvents

Anethole, anisaldehyde, linalool, phosphomolybdic acid, vanillin, cycloheximide, chloramphenicol, barium chloride, sulphuric acid and ketoconazole used were of analytical

grade and purchased from Sigma (Germany). Ethanol, ethyl acetate, toluen were of analytical grade and purchased from Kemika (Croatia), and nistatin was of analytical grade and purchased from Pliva (Croatia).

Microbiological media (Sabouraud 2%, *w/V*, glucose agar and broth) were purchased from Biolife (Italy).

Plant material, extract preparation and isolation of essential oil

The air-dried, powdered anise fruits obtained from a commercial source were extracted with 90% (*V/V*) ethanol and the fluid extract (1+1) was made. Anise fluid extract was kept at +4 °C in dark-glass containers until analysis.

The anise fruits were ripe and 100 g was submitted for 3 h to steam distillation in a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate (Fluka, Germany) and stored at +4 °C in dark glass containers.

Phytochemical investigation

Thin-layer chromatography (TLC) was applied for testing the presence of characteristic compounds (anethole, anisaldehyde and linalool) in the anise fluid extract and anise essential oil according to *European Pharmacopoeia* (13).

Briefly, 5 µL of reference solutions of anethole, anisaldehyde and linalool, together with 10 µL of investigated anise oil, were applied onto pre-coated silica gel F₂₅₄ plates with fluorescent indicator (10 x 20 cm, tickness 0.1 mm, Macherey-Nagel, Germany). Plates were developed in the solution of ethyl acetate and toluen (7+93, *V/V*), in a chamber saturated at least one hour before examination. After drying, plates were sprayed with freshly prepared vanillin reagent (1% of vanillin in 2% sulphuric acid in ethanol, *V/V*) and heated at 100 °C for 10 minutes. Chromatograms were then examined under daylight within 10 minutes.

Anethole was used as reference substance for identification of characteristic compounds in anise fruits. The same procedure was applied as for anise oil, with a slight modification. Plates were sprayed with freshly prepared phosphomolybdic acid solution (0.1% of phosphomolybdic acid in sulphuric acid 40+60, *V/V*) and heated at 120° C for 5 minutes.

Reference solution was prepared as a solution of 80 µL of anethole, 3 µL of anisaldehyde and 1 µL of linalool in 1 mL of toluene.

Microorganisms tested

Fungi used were isolates of yeasts from clinical specimens of blood, wounds or urine and dermatophyte strains from superficial mycoses of man. All the tested fungal species were from the Culture Collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia, and were determined according to morphological data described in literature (16, 17).

Before investigation, yeasts were cultivated on Sabouraud 2%-glucose agar (SGA) with supplement of 50 mg L⁻¹ chloramphenicol. Dermatophytes were cultivated on the

same agar with addition of 50 mg L⁻¹ cycloheximide to avoid contamination. All tested yeasts were cultivated for 72 h at 25 ± 2 °C and dermatophytes for 7 days for inoculum preparation.

Antifungal investigation

Antifungal activity of anise fluid extract and anise oil was tested by the agar diffusion method described in *European Pharmacopoeia* (13). Minimum inhibitory concentration (MIC) was determined using the serial two-fold broth dilution method described by Pepeljnjak *et al.* (3).

For the agar diffusion method, 25 ± 2 mL of sterile and melted SGA at 45–50 °C was inoculated with 1 mL of approximately 1–5 × 10⁶ CFU mL⁻¹ of yeast cells or conidia and mycelial structures of dermatophytes in sterile physiological saline in Petri dishes (9 cm). Inoculum density was measured with McFarland's standard solution of freshly prepared barium sulfate in sterile water; density of 0.01% BaCl₂ in 1% H₂SO₄ solution equals approximately 3 × 10⁸ cells mL⁻¹ (18).

After drying SGA at room temperature for a maximum of 30 minutes, holes of 6 mm in diameter were made with stainless steel cylinders and filled (60 µL) with the fluid extract or essential oil. One experiment was done in a Petri dish for one fungal strain. Plates were then incubated at +4 °C for 1 hour and at 25 ± 2 °C for 48 h for yeasts and 7 days for dermatophytes. After the incubation period, inhibition zones were measured and expressed in mm.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using the serial broth dilution method described by Pepeljnjak *et al.* (3). MIC was determined by the broth two-fold macro dilution method in Sabouraud 2%-glucose broth starting with 50% (V/V) of the fluid extract or essential oil. MIC is defined as the lowest concentration of extract or essential oil that allows no more than 20% growth of the fungi, visualized as a reduced number of colonies after removing the loop with approx. 10 µL of each dilution on SGA and incubation at 25 ± 2 °C for 72 h for yeasts and 7 days for dermatophytes. MFC is defined as the lowest concentration of the extract or essential oil that completely inhibited the growth of fungi.

Statistical analysis

Minimum inhibitory concentrations of the anise fruits extract and anise essential oil were compared using the Wilcoxon signed rank test (GraphPrism software for Windows, GraphPad Software, San Diego, USA). The level of significance was $p < 0.01$.

RESULTS AND DISCUSSION

In the anise fruits sample studied, anethole was proven to be present by qualitative TLC analyses, while anethole, anisaldehyde and linalool were found in the essential oil obtained by water vapor distillation from seeds.

The results of the antifungal effect of the fluid extract and essential oil of anise, together with referent antimycotics, are shown in Tables I and II. The fluid extract of anise

showed antifungal activity against all the species of dermatophytes investigated, with inhibition zones from 20 to 29 mm. The largest inhibition zone was observed against *T. mentagrophytes* (29 mm). The fluid extract of anise had a well-balanced antifungal effect on the fungi species *Candida albicans*, *C. tropicalis*, *C. pseudotropicalis* and *C. krusei*, with an inhibition zone of 10 to 12 mm. The largest inhibition zone was observed in *C. albicans* (12 mm). Species *C. glabrata* is resistant to the investigated fluid extract of anise, but it had a promotive effect on the growth of the species *Geotrichum* spp. It was observed that comparing the inhibition zones of fungi (excluding species *C. glabrata* and *Geotrichum* spp.) and dermatophytes, dermatophytes were more sensitive to the effect of the fluid extract of anise than fungi and their inhibition zones were on average 10 mm larger.

The essential oil of anise shows antifungal activity against all species of dermatophytes studied, with inhibition zones of 21 mm (*Microsporium gypseum*), 23 mm (*Trichophyton mentagrophytes*), 25 mm (*T. rubrum*) and the largest inhibition zone of 27 mm in *M. canis*.

All the fungi investigated are sensitive to the essential oil of anise. The largest inhibition zone was found in the species *C. parapsilosis* (30 mm), followed by *C. albicans* with a 29 mm inhibition zone, *C. glabrata* with a 21 mm inhibition zone, and *Geotrichum* spp. with an inhibition zone of 17 mm. It is interesting that the antifungal activity of aerosol, in other words, irreversible inhibition of the growth of fungi *C. tropicalis*, *C. pseudotropicalis* and *C. krusei* was observed both on the surface and in the depth of the agar during the experiment when the essential oil was dropped into the hole (60 µL) of the Petri dish: concentration of the essential oil aerosol in the Petri dish was 0.9 µL cm⁻³, with an incubation temperature of 25 ± 2 °C.

Data on minimum inhibitory concentrations and minimum fungicidal concentrations for the fluid extract and for the essential oil of anise are shown in Table II.

All the dermatophyte species studied were sensitive to the fluid extract and essential oil of anise. MIC of the fluid extract are between 1.5 and 9.0% (V/V), while those of

Table I. Antifungal activity of anise fluid extract and essential oil by the diffusion method

	Fungi	Inhibition zone (mm)		
		Reference substance ^a	Fluid extract	Essential oil
Yeasts	<i>Candida albicans</i>	18	12	29
	<i>C. tropicalis</i>	23	11	AE ^c
	<i>C. pseudotropicalis</i>	18	10	AE ^c
	<i>C. parapsilosis</i>	18	11	30
	<i>C. krusei</i>	17	11	AE ^c
	<i>C. glabrata</i>	18	0	21
	<i>Geotrichum</i> spp.	19	GPA ^b	17
Dermatophytes	<i>Trichophyton rubrum</i>	21	20	25
	<i>T. mentagrophytes</i>	24	29	23
	<i>Microsporium canis</i>	21	26	27
	<i>M. gypseum</i>	21	23	21

^a Nistatin as reference substance (0.5 mg mL⁻¹) for yeasts and ketoconazole for dermatophytes.

^b GPA – growth promotion activity.

^c AE – fungicidal influence of aerosol (0.7 µL cm⁻³).

Table II. Antifungal activity of anise fluid extract and essential oil by the dilution method

Fungi		Antifungal activity					
		Reference substance ^a (µg mL ⁻¹)		Fluid extract (% V/V)		Essential oil (% V/V)	
		MIC ^b	MFC ^c	MIC	MFC	MIC	MFC
Yeasts	<i>Candida albicans</i>	0.5	1	19	25	1.00 ^d	2.00 ^d
	<i>C. parapsilosis</i>	1	2	18	25	0.10 ^d	0.20 ^d
	<i>C. tropicalis</i>	1	2	18	25	0.10 ^d	0.20 ^d
	<i>C. pseudotropicalis</i>	0.5	1	17	20	0.20 ^d	0.39 ^d
	<i>C. krusei</i>	0.5	1	20	25	0.20 ^d	0.39 ^d
	<i>C. glabrata</i>	2	4	nt	nt	0.10 ^d	0.20 ^d
	<i>Geotrichum</i> spp.	2	4	nt	nt	1.56 ^d	3.12 ^d
Dermatophytes	<i>Trichophyton rubrum</i>	0.25	0.5	1.5	3	0.20 ^d	0.39 ^d
	<i>T. mentagrophytes</i>	0.50	1	9.0	20	0.78 ^d	1.56 ^d
	<i>Microsporum canis</i>	0.25	0.5	2.5	5	0.10 ^d	0.20 ^d
	<i>M. gypseum</i>	0.25	0.5	7.0	15	0.20 ^d	0.39 ^d

^a Nistatin as reference antimycotic against yeasts tested and ketoconazole against dermatophytes tested.

^b MIC – minimum inhibitory concentration.

^c MFC – minimum fungicidal concentration.

^d Significantly different from fluid extract ($p < 0.01$).

nt – not tested.

the essential oil are lower, below 1% (V/V), and range from 0.10 to 0.78% (V/V). The most sensitive dermatophyte is *M. canis* with MIC 0.10% (V/V) for the essential oil, and *T. rubrum* with MIC 1.50% (V/V) for the fluid extract.

MIC values for the fungi species studied are also below or equal to 1.56% (V/V). *Geotrichum* spp. has the highest MIC value of 1.56% (V/V), followed by *C. albicans* (MIC 1%, V/V), *C. pseudotropicalis* and *C. krusei* (MIC 0.20%, V/V), *C. parapsilosis*, *C. tropicalis* and *C. glabrata* (MIC 0.10%, V/V). For the fungi species that were sensitive to the fluid extract of anise, MIC values between 17 and 20% (V/V) were recorded. The most sensitive species is *C. pseudotropicalis* with MIC 17% (V/V), followed by *C. parapsilosis* and *C. tropicalis* with MIC 18% (V/V), *C. albicans* with MIC 19% (V/V) and *C. krusei* with the highest MIC of 20% (V/V).

Comparison of the inhibition zones of the fluid extract and essential oil of anise shows a stronger effect of the anise essential oil on the species *C. albicans*, *C. parapsilosis* and *C. glabrata* compared to the reference substance. The antifungal activity of the anise essential oil is weaker than that of nistatin in *Geotrichum* spp. The antifungal activity of the fluid extract of anise is weaker compared to the referent substance. It is interesting that the antifungal activity of the fluid extract and essential oil of anise is higher or equal to the antifungal activity of the referent substance (ketoconazole). It is also very interesting that during the experiment (lasting up to 7 days for dermatophyte) the essential oil was fungicidal, with an inhibition zone between 21 and 27 mm, which implies that both components with low volatility and stable components are present in the anise essential oil.

Comparing the MIC values for the referent substances nistatin and ketoconazole and MIC values for the fluid extract and essential oil of anise, we can come to the con-

clusion that the referent substances demonstrate much lower values of MIC. But, differences in MFC values are also evident between the essential oil and the fluid extract of the fungi *Candida* species as well as of the dermatophytes studied. The statistically significant difference of MIC values ($p < 0.01$) between the essential oils and the fluid extract of anise implies that very potent fungicidal components are present in the essential oil of anise, which are not present in the fluid extract or are present in very low concentrations.

In their detailed investigations, Hammer *et al.* (19) demonstrated the results on the antimicrobial effect of different essential oils on Gram-positive and Gram-negative bacteria, as well as on the fungus *Candida albicans*. Among other essential oils studied, they determined the MIC value for the essential oil of anise, which was between 0.25 and 2.0% (V/V) for the bacteria: *Acinetobacter baumannii*, *Aeromonas sobria*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella thyphimurium*, *Serratia marcescens* and *Staphylococcus aureus*, while for the yeast *Candida albicans* the MIC value was 0.5% (V/V). Our investigations have shown higher MIC values of the essential oil for the species *C. albicans*, which is 1.0 % (V/V) (for other clinical fungi isolates the range is between 0.10 and 1.56%, V/V). The differences in MIC values can be explained by different concentrations of antifungal components in the essential oil of anise, but also in the application of methods for determining MIC values. This is why data obtained by Hammer and coworkers cannot be compared to our data because they used a dilution of the essential oil in agar and we used a dilution in broth. MIC values are also influenced by a number of variables, such as the composition of the culture medium, inoculum density, *etc.* (20).

Gülçin *et al.* (21) determined the antimicrobial effect of the water extract of anise seeds but not that of the ethanol extract. The antimicrobial activity of the anise extract was investigated on Gram-positive bacteria *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, *Micrococcus luteus* and Gram-negative bacteria *Proteus mirabilis*, *Citrobacter koseri*, *Enterobacter aerogenes*. The extracts showed no bactericidal effect on *Pseudomonas aeruginosa* and *Escherichia coli*. The antifungal activity of water and ethanol extracts of anise against the fungus *Candida albicans* was investigated, but fungicidal effect was observed only in the ethanol extract, with a small inhibition zone of 8 mm. Our investigations have also shown an antifungal effect of the ethanol extract of anise with an inhibition zone of 12 mm for the ethanol extract and 29 mm for the essential oil, and MIC values of 19% (V/V) for the ethanol extract and 1 % (V/V) for the essential oil (Tables I and II). Stronger antifungal activity of the essential oil compared to the fluid extract is probably due to the higher concentration of *trans*-anethole in the essential oil.

CONCLUSIONS

The antifungal activity of the fluid extract and essential oil of anise on clinical isolates of fungi and dermatophytes was tested *in vitro*. The essential oil of anise showed a more potent antifungal activity against all fungi studied compared to the fluid extract of anise. The essential oil of anise also inhibited the growth of dermatophytes in lower concentrations than the fluid extract of anise. Further investigations should be performed on a wider range of fungi and dermatophytes in order to determine which component of the essential oil (*trans*-anethole, anisaldehyde, estragole, anisketone) shows the most po-

tent antifungal activity. The antifungal activity could also be a result of the synergism of its components. In addition, the antifungal activity against yeasts of the aerosol of the essential oil of anise and its main components should be investigated.

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S A Ž E T A K

Antimikotsko djelovanje tekućeg ekstrakta i eteričnog ulja iz plodova aniša (*Pimpinella anisum* L., *Apiaceae*)

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Ispitana je antimikotsko djelovanje tekućeg ekstrakta i eteričnog ulja iz plodova aniša (*Pimpinella anisum* L., *Apiaceae*) na kliničke izolate sedam vrsta kvascima sličnih gljivica i četiri vrste dermatofita. Za ispitivanje antifungalne aktivnosti upotrebene su metode difuzije cilindrima i metode razrjeđivanja u tekućem hranilištu. Tekući ekstrakt aniša pokazuje antifungalnu aktivnost na vrste *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis* i *C. krusei* s vrijednostima minimalne inhibitorne koncentracije (MIK) između 17 i 20% (V/V). Vrsta *C. glabrata* je rezistentna na djelovanje ekstrakta, a uočeno je promotivno djelovanje ekstrakta na rast *Geotrichum* sp. Ekstrakt aniša pokazuje inhibiciju rasta dermatofita (*Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis* i *M. gypseum*) s MIK vrijednostima između 1,5 i 9,0% (V/V). Eterično ulje aniša pokazuje jaku antifungalnu aktivnost na kvascima slične gljivice s vrijednostima MIK-a nižima od 1,56% (V/V), a na dermatofite s vrijednostima MIK-a nižima od 0,78% (V/V). Utvrđena je značajna razlika ($p < 0,01$) između MIK-a tekućeg ekstrakta aniša i aniševog eteričnog ulja. Eterično ulje aniša pokazuje jaču antifungalnu aktivnost na kvascima slične gljivice i dermatofite s vrijednostima MIK-a između 0,10 i 1,56% (V/V).

Ključne riječi: aniš, plod aniša, *Pimpinella anisum* (*Apiaceae*), tekući ekstrakt, eterično ulje, antifungalno djelovanje, *Candida*, dermatofit

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