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Method to Study Antimicrobial Effects of Essential Oils: Application to the Antifungal Activity of Six Moroccan Essences

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ABSTRACT

The Micro-atmosphere method of Kellner and Kober was modified and used to study antifungal properties of six essential oils that have different chemical compositions (three chemotypes from mugwort, *Artemisia herba alba,* one from thyme, *Thymus capitatus,* one from rosemary, *Romarinus officinalis* and one from Eucalyptus, *Eucalyptus globulus*). They were tested against 39 mold strains (13 from the genus *Penicillium,* nine from *Aspergillus* and 17 others). The essential oil from thyme was the most effective, suscessively followed by those from mugwort, rosemary and eucalyptus. The strains studied were classified into three groups: sensitive, intermediate and resistant.

Essential oils (EO) have been frequently reported to be antimicrobial agents. The best known in this field is thyme EO (*1,22*). Antibacterial (*6,8,11,18*) and/or antifungal (*5,18,19,20*) properties are well established for many other EO. Many practical uses of these properties have been suggested in various fields such as human (*7,20*) and animal (*8*) therapy and general sanitation. The raw material for perfumery is mainly made from EO. Some of the properties of their natural or synthetic components (deodorant properties) are due to their bacteriostatic activity (*3*). The "Paragerm" used in some hospitals for sanitation, particularly in surgical rooms is made from EO of high antibacterial and antifungal activities (*15*). Moreover, it has been reported that EO have interesting inhibitory effects against bacterial development in food products (*4*).

Morocco is a country rich in aromatic and medicinal plants. Most of these are used in the Moroccan folk tradition for many purposes. Perwincle (*Vinca minor*) and elecampane (*Inula viscosa*) are used to preserve cereal grains. Musky bugle (*Ajuga iva*), thyme (species from the genus *Thymus*), rosemary (*Romarinus officinalis*) and mints (species from the genus *Mentha*) are added to butter and other foods to improve their shelf-life. Laurel (*Nerium oleander*), pennyroyal (*Mentha pulegium*) and thyme are used to preserve dried figs. Many infectious diseases are cured or prevented by aromatic plants or their extracts. Thyme, rosemary, mugwort and various species of mint are used for open wounds and against various cutaneous infections.

To understand the scientific basis of these traditional practices, we carried out a study of the inhibitory effects of EO from some Moroccan aromatic plants against a number of mold species frequently involved in food spoilage. For this purpose, we modify the method of Kellner and Kober. This modified technique can be applied to study the general antimicrobial properties of EO.

MATERIALS AND METHODS

Essential oils used

Six EO were tested. They were extracted from aromatic plants chosen among the most widespread ones in the Moroccan territory. These plants also present some variable chemical profiles: phenol EO (thyme, *Thymus capitatus*), oxide EO (rosemary, *Romarinus officinalis* and eucalyptus, *Eucalyptus globulus*) and ketonic EO (mugwort, *Artemisia herba alba*). In the latter instance, we chose three EO belonging to three different chemotypes among the known eight in this species (*2*) that differ one from another in the nature of the main ketonic compounds (α-thujone, β-thujone or l-camphor).

Extraction of the EO was done in our laboratory by steam distillation, using a Clevenger apparatus as modified by Miquel et al. (*16*). EO were analysed by gas liquid chromatography (GLC), under the following conditions: (*a*) apparatus: HP 5730 A, with HP 3380 A integrator; (*b*) detector: flame ionization; (*c*) column: glass capillary 30 m length, 0.3 mm in diameter; (*d*) temperature: injection port: 220°C; detector: 220°C; column: programmed temperature, 80°C during 8 min and up to 180° for 4°C/min, then 180°C during 20 min; (*e*) carrier gas: nitrogen, flow: about 1 ml/min.

For quantitative analysis, the response factor is the same for all the compounds, as it is generally true for products of similar structure such as the monoterpenic substances.

Qualitative identification of the main components was done by combining preparative GLC and IR spectrometry methods according to Jennings (*9*) and Toulemonde (*23*). We also used chromatographic retention times determined under the conditions described above, and comparison with standard substances.

Due to the technique adopted in this work, based on the steam phase action, analysis of these vapors was done using the head space method. Five ml of EO was poured into a cylindric vial of about 100 ml (7 cm hight, 15 cm^2 section) which was then hermetically closed. The equilibrium was supposed to be established after 24 h. Then we pierced

the rubber cork with a hypodermic syringe and took 5 ml from the atmosphere contained in the vial at about 5 cm above the liquid surface. This volume was entirely injected into the chromatograph under the same conditions described above, except for the column. For head space analysis, we used a metallic column of 3 m length and 3 mm ID packed with FFAP at 5% on chromosorb WAW-60-80 mesh. The carrier gas flow was 15 ml/min.

The relative rate of each component of the vapor phase was determined as described above. An approximative global concentration of the EO in the head space at the equilibrium state was obtained by summing the surfaces of detectable peaks observed:

$$Cvp = \frac{\Sigma Avc}{\Sigma Alc} \times \frac{Atv.c}{Atl.c} \times \frac{Vl.c}{Vv.c} \times Cs$$

with:

Cvp =	global concentration of the vapor phase
ΣAvc =	the surface of the detectable peak in the chromatogram of the vapor phase
ΣAl.c =	the surface sum of the detectable peak in the chromatogram of the liquid phase
Atv.c =	Attenuation used in the injection of the vapor phase
Atl.c =	Attenuation used in the injection of the liquid phase
Vl.c =	Volume of the injected solution to get the chromatogram of the liquid phase = 2 µl
Vv.c =	Injected volume to get chromatogram of the vapor phase = 5 µl
Cs =	the concentrtion of the solution of the E.O. used to get the chromatogram of the liquid phase = 5% E.O. in hexane.

Strains of molds

Thirty-nine strains were used including 13 penicillia, 9 aspergilli and 17 others. Most of these strains belong to species important in food microbiology. Some of them are used to prepare fermented foods (*Geotrichum candidum* and *Penicillium caseicolum* in cheese), others are responsible for food spoilage, especially during storage (*Aspergillus niger, Penicillium italicum, P. digitatum, Alternaria tenuissima, Mucor spinosis*), others are dangerous to human and animal consumers because of the mycoses (*A. fumigatus*) or mycotoxicoses due to the toxins they can produce in foods and feeds (*A. flavus, A. ochraceus, P. cyclopium, Byssochlamys nivea.*

Test for antifungal study

Basic method. The technique used in this study is derived from the the microatmosphere method of Kellner and Kober. This consists of cultivating the microorganisms to be tested in petri dishes on agar medium, and incubating these dishes in reversed position after laying down the essential oil on a filter paper in the middle of the dish cover. The oil evaporates in the atmosphere of the dish and the volatile phase can exert its inhibitory effect on the inoculated microorganisms.

Technique. Using the principle described above, we set up the following method: pyrex glass petri dishes were used, with exactly the same shape (9.2 cm diameter, 1.5 cm height). An exact volume of 15 ml of Czapek-Dox Agar medium (pH 7.2) was poured into each dish, leading to an identical internal atmosphere volume in all the dishes.

Conidiospores from mold strains previously grown on the same medium at 25°C for 7 d, were suspended in a NaCl solution (0.9%) containing 0.01% Tween 80, and then were inoculated into the petri dishes (3 to 6 per dish) in radial lines (Fig. 1). The petri dishes were turned upside down and a 2-cm diameter filter paper (Macherey-Nagel MN713) was put in the middle of the cover and soaked with variable amounts of EO. For each 3-6 strains series, and for each oil, six petri dishes were prepared with 0 (control), 5, 10, 20, 50 and 100 µl, respectively, of EO. Small amounts, none leading to complete soaking of the filter paper, were completed up to 50 µl with ethanol. Thus a seventh petri dish (ethanol control) was prepared under the same conditions, with only 50 µl ethanol. The petri dishes were incubated in reversed position for 6 d, at room temperature (20-22°C). The petri dishes were not wrapped, thus foam incubation was not done to avoid false results due to evaporation of the oils in the foam atmosphere.

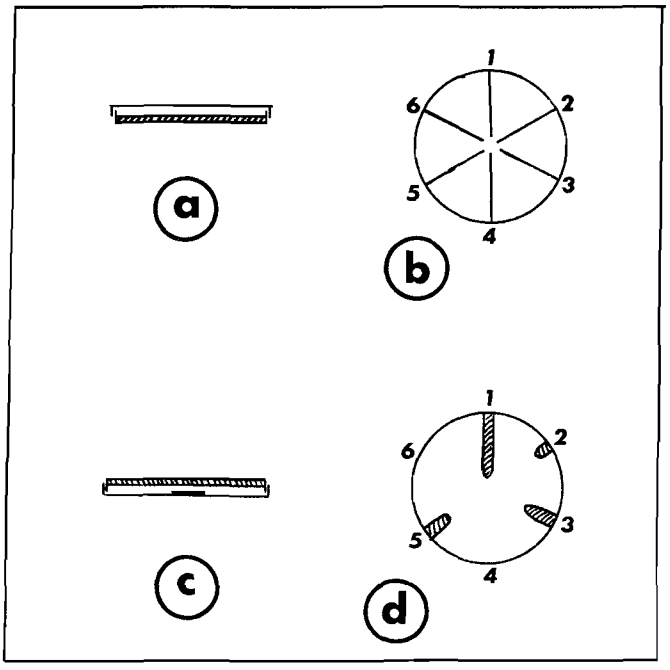


Figure 1. *The microatmosphere method of Kellner and Kober as modified by us. a, petri dish (9.2 cm diameter, 1.5 cm height) containing 15 ml of Czapek-Dox Agar; b, radial lines, inoculation of six molds; c, petri dish is turned upside down, a filter paper (2 cm in diameter) is put in the middle of the cover, and soaked with variable amounts of essential oil; d, after incubation (room temperature, 6 d), total or partial inhibition of growth can be observed.*

Expression of results. The partial inhibitory effect of EO was determined after incubation by comparison of the growth observed (length and width) to that of the control. The minimal amount of the oil able to completely inhibit each strain can be also determined under our experimental conditions. From these observations, the EO can be classified according to their antifungal effects, and the strains with regard to their sensitivity.

To clarify the expression of the results, a simplified method is suggested in which only the minimal amount of each EO causing total inhibition is taken into account: the degree of effectiveness of the oil (i.e. degree of sensitivity of the strain) is 5 when total inhibition is obtained with 5 µl EO, the degree is 4, 3, 2, 1 or 0 when total inhibition is reached respectively with 10, 20, 50, 100 or more than 100 µl EO.

Following this procedure, summing up the scores obtained for a given strain with the six oils, we arrive at a number, A, that represents the over-all degree of sensitivity of this strain; the higher this number is, the more sensitive the strain. On the other hand, summing up the scores obtained for a given EO with the 39 strains, we arrive at another number, B, that expresses the overall antifungal efficiency of the EO concerned; the higher this number is, the more effective the oil.

RESULTS AND DISCUSSION

Composition of the EO studied

Results of the EO analysis are reported in Table 1. Liquid EO analysis shows that the mugworts studied were from three different chemotypes: mugwort 1 was of the α-thujone chemotype (65% α-thujone), mugwort 2 of the β-thujone chemotype (83% β-thujone), mogwort 3 of the α-thujone and camphor chemotype (35% and 44%, respectively). The thyme EO analyzed had a typical carvac-

role profile (78%), while 1, 8 cineole was the main component in both eucalyptus and rosemary EO (69% and 52%, respectively), followed by camphene in the former (29%) and camphor for the latter (28%).

Head space analysis led to very different results, generally with higher concentrations for the lightest components (α-pinene, camphene) and smaller amounts for the heaviest ones (carvacrole and camphor).

Advantages of the technique described

Compared to the original method of Kellner and Kober, the technique described here has some advantages. First, it is inexpensive since it allows the study of several strains using the same petri dish. Second, the radial inoculation offers the advantage of easily estimating the inhibition effect by measuring the extent of growth and thus, to compare several strains under the same conditions for their sensitivity.

Comparative antifungal effectiveness of the essential oils

Comparing the over-all degrees of activity obtained for the oils studied (Fig. 2), it can be observed that thyme oil was the most effective (B = 175), followed far after by the three mugwort oils (B = 74-77) and then by rosemary and eucalyptus (B = 41 and B = 28, respectively). Moreover, considering the individual effectiveness of the oils on each microorganism, it can always be written: Thyme ≥ mugwort ≥ rosemary and eucalyptus (≥: more effective, or having a similar degree of effectiveness).

This relation is the same as that expected if the antifungal properties of the oils were due to their main components. It is now well established that phenols are more effective than ketones, and these latter are more active than oxides (*7,13*). Our results confirm these observations, since the essential oil of thyme we used had a high carvacrole, and those from mugwort had high ketone level (α-thujone, β-thujone and camphor), while 1, 8 cineole is the major component in both rosemary and eucalyptus oils. However, other miscellaneous components may have a relatively important effect, since the composition of the vapor phase which is involved in our experiments is rather different from that of the liquid phase. Thus, some components weakly represented in the liquid phase, are more important in the vapor phase, like α-pinene and camphene in the three mugwort chemotypes, camphene in eucalyptus and rosemary, paracymeme and γ-terpinene in thyme (see Table 1). Also, the difference between the over-all effectiveness of rosemary oil (B = 41) and that of eucalyptus oil (B = 28) may thus be explained. Moreover, the similar over-all effectiveness of the three mugwort oils may be explained in spite of their different major components (α-thujone, β-thujone and camphor), because all these are monoterpenic ketones that have almost the same effectiveness (*13*). In addition, these three oils have very few differences in minor components.

On the other hand, the low concentrations of phenols in the thyme vapor phase must be taken into account; carvacrole is replaced as the most important component by γ-terpinene, which is less active, and by paracymene, which is more effective than the terpinene (*13*). However, thyme essential oil remains highly effective, more so than all the other studied.

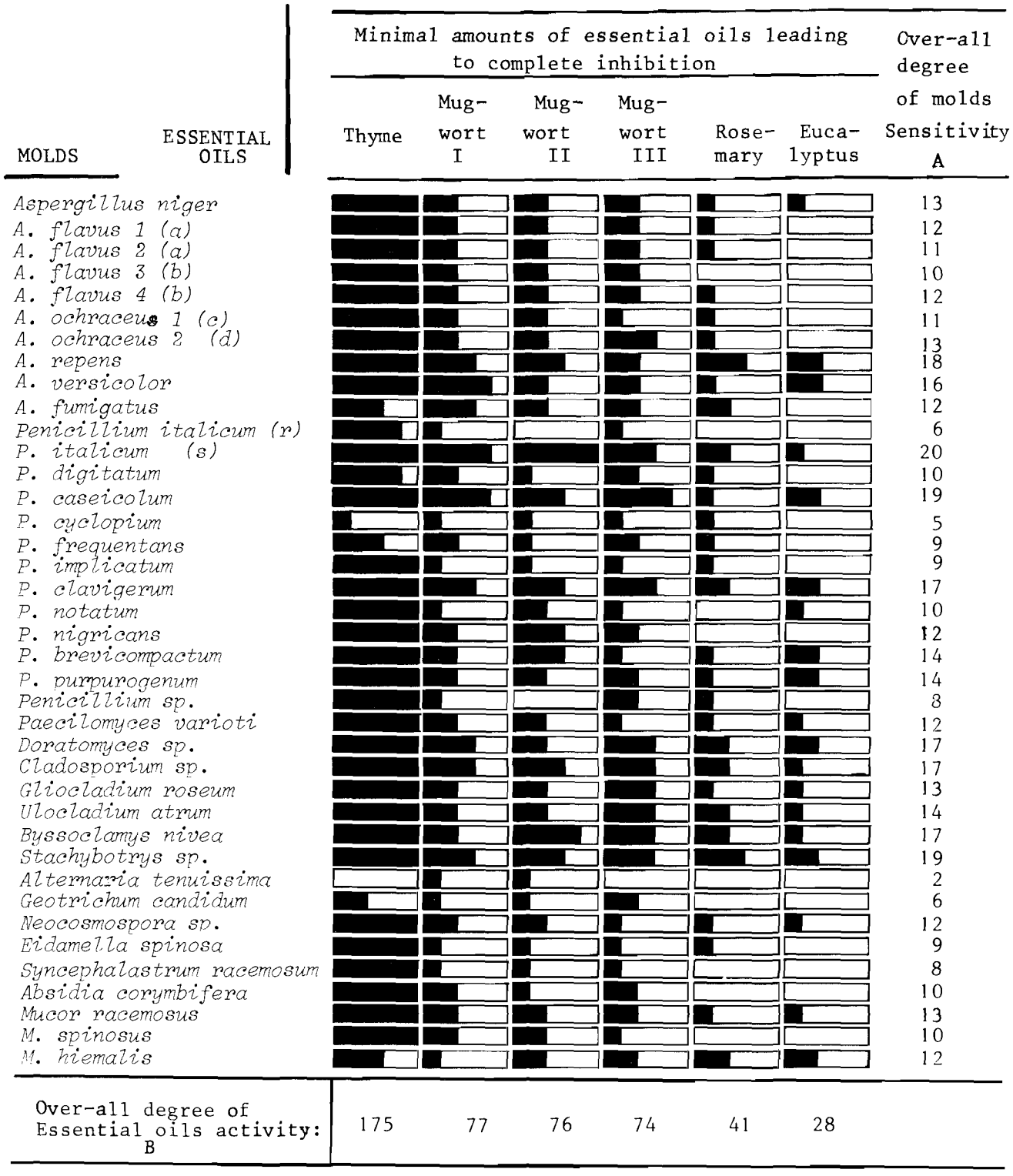
Sensitivity of the microorganisms studied

The microorganisms studied can be classified into 3 groups (Fig. 2).

(*a*) Microorganisms highly sensitive to all the essential oils studied (17≤A≤20) include *Aspergillus repens, Penicillium clavigerum, P. italicum* "s", *Stachybotrys* sp., *B. nivea* and *Doratomyces* sp. *P. caseicolum* (A = 19) and *Cladosporium* sp. (A = 17) also have a high

TABLE 1. *Composition of the six essential oils studied. A: Liquid essential oils composition, B: Composition of the steam phase (head space). (Results are given in % total essential oil).*

Plant		Essential oil										
		α-pinene	camphene	β-pinene	paracymene	γ-terpinene	1,8 cineole	α-thujone	β-thujone	Camphor	Carvacrole	Unknown
Mugwort I	A	1	2	-	-	-	1.5	65	8	8	-	-
	B	13	20	-	-	-	14.1	39	39	9	-	-
Mugwort II	A	3	3	-	-	-	4.5	traces	83	6	-	
	B	22	12	-	-	-	6	traces	28	2	-	-
Mugwort III	A	2	7	-	-	-	5.5	35	8	38	-	-
	B	9	20	-	-	-	9	17	17	21	-	21
Thyme	A	traces	1.5	traces	15	1	-	-	-	-	78	-
	B	-	-	-	27.5	44	-	-	-	-	0.9	-
Eucalyptus	A	-	29	-	-	-	69	-	-	-	-	-
	B	-	39	-	-	-	58	-	-	-	-	-
Rosemary	A	15	4	6	-	-	52	-	-	12	-	-
	B	39	18	10	-	-	37	-	-	3	-	-



a: non-aflatoxin producing - b: aflatoxin producing-
c: ochratoxin producing - d: non-orchratoxin producing -
r: resistant to 10 ppm thiabendazole on citrus fruits -
s: sensitive to 10 ppm thiabendazole on citrus fruits.

Figure 2. *Antifungal efficiency of the essential oils studied.*

over-all sensitivity, while being very resistant to a given essential oil (rosemary and eucalyptus, respectively).

(*b*) Highly resistant microorganisms, having a weak over-all sensitivity ($A<10$), with generally a medium to very weak individual sensitivity to thyme, and weak to very weak individual sensitivity to the other oils include *Penicillium frequentans* ($A=9$), *P. cyclopium* ($A=5$) and *A. tenuissima* ($A=2$). Four other strains, namely *Penicillium* sp., *P. implicatum, Eidamella spinosa* and *Syncephalastrum racemosum,* have a similar over-all sensitivity ($A<10$), but they are highly sensitivie to thyme oil.

(*c*) Microorganisms with a medium over-all sensitivity ($10<A<17$) with, however, a very high sensitivity to thyme oil. All the other strains belong to this group.

Influence of the amount of essential oil

To be able to estimate the inhibitory effect of each essential oil, it is necessary to know the total oil concentration in the phase directly in contact with the microorganisms. Under our experimental conditions, this phase is the petri dish atmosphere, the composition of which is very difficult to determine. For this reason, we supposed that the petri dish atmosphere composition is similar to that of the vapor phase in equilibrium with the liquid phase while the oil is left in a hermetic vial at room temperature for 48 h. Following this hypothesis, we analyzed the head space of the oils studied and obtained the following results: the concentrations were about 10^{-6} to 10^{-5} (v/v) for thyme oil, 10^{-5} for mugwort (three chemotypes) and 10^{-5} to 10^{-4} for rosemary and eucalyptus. These concentrations are maxima which cannot be reached in the petri dish atmosphere, because of the relative humidity due to water evaporation from the nutritive medium.

Moreover it must be emphasized that petri dishes are not hermetic. That is probably why the influence of the amount introduced in each dish is important; the larger this amount is, the closer we must be to the saturation point.

On the other hand, one can suppose that even if saturation is reached with small amount of the essential oils (5 μl), this saturation is maintained for less time than those due to higher amounts (10, 20, 50 or 100 μl). In other words, the differences observed among various amounts of oil may be actually due to various durations of contact between the microorganisms and the essential oil.

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