Evaluation of Inhibitory Data of Essential Oil Constituents Obtained with Different Microbiological Testing Methods

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Abstract

In the past a number of different testing methods have been elaborated in order to investigate the antimicrobial properties of essential oils and their constituents. However, the gained results obtained with different testing set-ups are limited, because they are strongly dependent from the applied experimental conditions and cannot be compared in most cases.

To demonstrate the data variety, inhibitory data of eugenol - one of the best examined compound found in essential oils - from own investigations and from literature against *Eschericia coli* were chosen as an example and evaluated after compilation.

As a result from the critical discussion methodical parameters are outlined for testing the antimicrobial activity of essential oils and their constituents in order to improve the comparability of the obtained results.

Introduction

In vitro examinations of essential oils and of their constituents on their growth inhibiting properties against microorganism have been done in the past by use of different testing methods. Results obtained by these methods strongly depend on the applied experimental conditions, and therefore, their comparability is impaired in most of the cases. An evaluation of the antimicrobial efficiency of an examined compound becomes more or less complicated by these influences. To demonstrate the data variety, inhibitory data of eugenol -- one on the best examined constituent found in essential oils -- against *Eschericia coli* resulting from different test methods were chosen and compiled together including results from own investigations.

Results

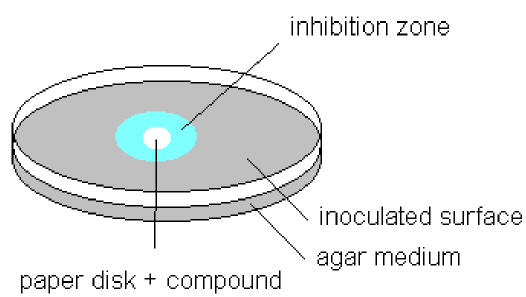
Eugenol inhibited growth of *E. coli* in all test systems used. With none of the different types of test methods strictly constant inhibitory data are reported.

Inhibitory zone formation is observed either with the paper disk technique (Tab. 1, Fig. 1) or hole technique (Tab. 2, Fig. 2) in agar diffusion test with eugenol. The size of the inhibitory zones -- often taken as a measure for the efficiency of a compound -- ranged from 12 to 26 mm. In comparison to neomycin, eugenol was more active by this method.

Tab. 1 Inhibitory Data of Eugenol Obtained in the Agar Diffusion Paper Disk Test

Strain	Inhibition zone	Agar	Compound dose	Incubation time/ T in °C	Solubility enhancer	Reference compound	Author
unspecified	12 mm	blood	moistened disk ?	18 h/ 37	none	none	Suresh et al., 1992
unspecified	13 mm	malat	9 mm disk, 20 µl	18 to 24 h/ 30	none	none	Weigand, 1986
unspecified	20 mm	Difco Pennassay base, seed agar	moistened disk ?	24 to 72 h/ 37	EtOH, acetone	none	Nadal et al., 1973
unspecified	21 mm	tryptone-yeast-glucose	9.5 mm disk, 2 µl	18 to 24 h/ 37	EtOH	none	Morris et al., 1979
unspecified	25 mm	nutrient, potato-dextrose	moistened ? 10 mm disk	cited	Tween ?	none	Megalla et al., 1980

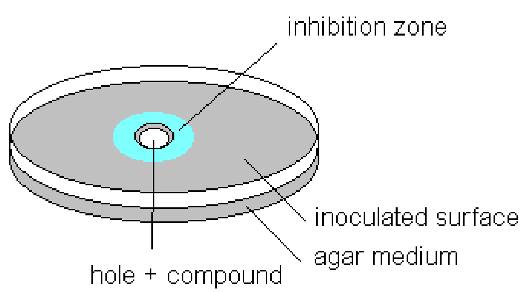
Fig. 1 Model of the Agar Diffusion Paper Disk Test



Tab. 2 Inhibitory Data of Eugenol Obtained in the Agar Diffusion Hole Test

Strain	inhibition zone	Agar	Compound dose	Incubation time/ T in °c	Solubility enhancer	Reference compound	Author
NCIB 8879	18.5 mm	Iso-sensitest	4 mm hole, 10 µl	48 h/ 25	none	none	Deans et al., 1989
unspecified	26 mm	nutrient	10 mm hole, 100 µl	72 h/ 30	none	none	Dey, 1984
unspecified	1.2-fold rel. to neomycin	Diagnostic sen-sitivity test	7 mm hole, ? µl	24 h/ 27	Tween 20, PEG 400, DMSO	neomycin	Laekeman et al., 1990

Fig. 2 Model of the Agar Diffusion Hole Test

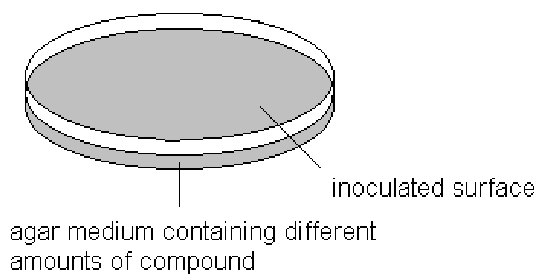


Results obtained with the agar dilution technique revealed growth inhibition by eugenol at 10 and 500 mg/l (Tab. 3, Fig. 3).

Tab. 3 Inhibitory Data of Eugenol Obtained in the Agar Dilution Test

Strain	MIC in mg/l	Agar	Inoculum size	Incubation time/ T in °C	Solubility enhancer	Reference compound	Author
UPR	10	nutrient	not given	24 to 72 h/ 37	EtOH, acetone	none	Nadal et al., 1973
unspecified	500	tryptic soy	10 E5/ml	cited	Tween 80, acetone	none	Prudent et al., 1993
unspecified	500	cited	cited	cited	cited	cited	Katayama et al., 1960

Fig. 3 Model of the Agar Dilution Test



MIC data obtained with eugenol in serial dilution tests ranged from 63 to 600 mg/l (Tab. 4, Fig. 4), and MMC data from 500 to 3000 mg/l (Tab. 5, Fig. 4). Comparison of the relative efficiency of eugenol has been done with phenol, naladixinic acid sodium salt, and hexachlorophen.

Tab. 4 Minimal Inhibitory Concentrations of Eugenol Obtained in the Serial Dilution Test

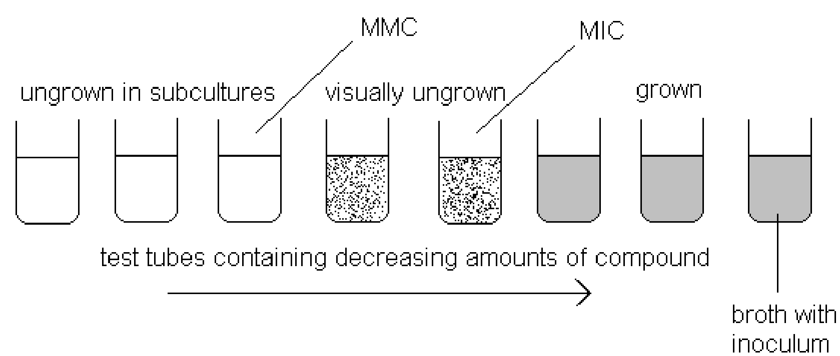
Strain	MIC in mg/l	Broth	Inoculum size	Incubation time/ T in °C	Solubility enhancer	Reference compound	Author
unspecified	63	nutrient	10 E5/ml	48 h/ 30	DMSO, EtOH	none	Jay et al., 1984
K12 Wildtype ATCC 23716	296 (= ED_{50})	meat extract, casein peptone	adjusted by optical density	4 h/ 37	without ?	phenol	Barelmann, 1994
NCTC 363	390	nutrient	microbial suspension	48 h/ 37	Tween 20	none	Yousef et al., 1979
ATCC 9673	400	nutrient, glucose, yeast extract	adjusted by optical density	48 h/ 37	DMF	none	Kubo et al., 1991
unspecified	250 - 500	cited	cited	cited	acetone	phenol, hexa-chlorophen	Weuffen et al., 1970
unspecified	500	tryptone-yeast-glucose	300000 viable organisms	18 to 24 h/ 37	EtOH	none	Morris et al., 1979
ATCC 11229	500	nutrient	microbial suspension	24 h/ 37	none	none	Maruzzella et al., 1961
ATCC 25922 mut. IV	550	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	Naladixinic acid Na, phenol	Pauli, 1994
ATCC 25922	550	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	phenol	Pauli, 1994
clinical isolate	600	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	phenol	Pauli, 1994
ATCC 25922	4000 *	63% sucrose, bovine serum	10 E7/ml	2 min./ 37	PEG 400	none	Briozzo et al., 1989

* 1.4 E3/ml survivors

Tab. 5 Minimal Microbicidal Concentrations of Eugenol Obtained in the Serial Dilution Test

Strain	MMC in mg/l	Broth	Inoculum size	Incubation time/ T in °C	Solubility enhancer	Reference compound	Author
K12 Wildtype ATCC 23716	500 (approx.)	meat extract, casein peptone	adjusted by optical density	4 h/ 37	without ?	phenol	Barelmann, 1994
ATCC 25922	700	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	phenol	Pauli, 1994
ATCC 25922 mut. IV	750	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	Naladixinic acid Na, phenol	Pauli, 1994
NCTC 363	780	nutrient	microbial suspension	48 h/ 37	Tween 20	none	Yousef et al., 1979
clinical isolate	800	meat extract, casein peptone	100 - 300 CFU /ml	18 h/ 37	Tween 20	phenol	Pauli, 1994
unspecified	1000	Merck, Standard 1-Nährbouillon	microbial suspension	24 h/ 37	ultrasonic	phenol	Münzing et al., 1980
unspecified	1000	cited	cited	cited	acetone	hexachlorophen, phenol	Weuffen et al., 1970
CCM 180	3000	mercapto acetate	10 cells/nl	cited	DMSO	none	Zemek et al., 1979

Fig. 4 Model of the Serial Dilution Test



Eugenol vapors are capable to inhibit growth of *E. coli* (Tab. 6).

Tab. 6 Inhibitory Data of Eugenol Obtained in Vapor Phase Activity Tests

Strain	Inhibition	Agar, Inoculation	Compound dose	Incubation time/ T in °C	Solubility enhancer	Reference compound	Author
unspecified	+++	dextrose, *E. coli* on surface	4 drops, inverted plate	24 h/ 37	none	none	Kellner et al., 1955
ATCC 11229	25 mm	nutrient, *E. coli* on surface	0.5 ml, inverted plate	48 h/ 37	5% glycerol	none	Maruzzella et al., 1961
IAM 1239	surface: no growth	agar included *E. coli* cells	20 mg, chamber	24 h/ 37	none	none	Gocho, 1991

Discussion

Results obtained with the agar diffusion test depend -- beside the inhibitory activity of a test compound -- on the ability of the test compound to move through the agar medium. Diffusion rates of essential oil constituents in the agar medium are generally unknown, and therefore, quantitative comparison of inhibitory zones, as usually done with antibiotics, is critical (see Tab.1). Inhibitory data of eugenol varied markedly, which can be explained through the influence of various factors (1), e.g., disk size, amount of compound applicated to disk, agar type, agar content, pH, volume of agar, and type of microbial strains used.

The agar dilution test requires 75 mg amount of compound for four dilutions steps (2000, 1000, 500, 250 mg/l) by use of agar layers with 20 ml volume. In contrast, the need of compound at the 2000 mg/l dose is 100-times lower in the serial dilution test with 12 dilution steps as it was used in the own examinations. In addition, the serial dilution test allows to compare inhibitory data with well-known antimicrobials, e.g., phenol or naladixinic acid sodium salt, an actual antibiotic compound. The deviations of MIC and MMC data of eugenol against *E. coli* were tolerable: in the mean MIC values ranged between 250 to 600 mg/l and MMC between 500 and 1000 mg/l, which seem to be a consequence of different test modifications used in the listed examinations. Therefore, it appears to be promising to consider a standardization of this method for testing essential oils and their constituents.

Conclusion

Among the methods used to examine the in vitro antimicrobial activity of essential oil components, the serial dilution technique appears to be the most promising one. This method yields two types of inhibitory data, the MIC and MMC from subcultures. Additionally, this method allows to work with very low amounts of test substances and enables comparison with clinically relevant antibiotics.

A standardized serial dilution test should reflect the following factors:

1) Registered microbial strains
2) Controlled counts of inoculated micro-organisms
3) Appropriate culture media for each type of micro-organisms
4) Defined inoculation time and inoculation temperature
5) Appropriate solubility enhancer, e.g. Tween 20
6) Evaluation and control of inhibitory data by use of antibiotics
7) Testing of short-time inhibitory activity
8) Recording of ED_{50} inhibitory data

Experimental

Microbial strains: *E. coli* ATCC 25922, *E. coli* ATCC 25922 mutant IV, *E. coli* clinical isolate

Test tubes: microtiter plates contained a series of 12 test tubes with a volume of 300 µl per tube. Final liquid volume was 250 µl.

Compound dilution: after dilution each test tube contained 80% of the compound dose of the previous test tube.

Inoculation: *E. coli* strains were grown in Mueller-Hinton bouillon overnight at 37 °C. The diluted microbial suspension contained 100 to 300 colony forming units (CFU) per ml, of which 25 µl were added to each test tube.

MIC and MMC determination: minimal inhibitory concentration (MIC) was taken from the concentration of the lowest dosed test tube showing visually no growth. 10 µl from each visually ungrown test tube were subcultured on Mueller-Hinton agar. The minimal microbicidal concentration (MMC) was taken from the concentration of the lowest dosed test tube showing no growth in subculture.

Control of inhibitory data: the experimental conditions, such as bacterial count and test medium, were appropriate to reproduce inhibitory data of a known antibiotic (naladixinic acid sodium salt) against *Eschericia coli*. The antibiotic was used as reference compound in each test series. Further control of obtained data was done by CFU/ml determination.

References

1) **JANSSEN, A.M., SCHEFFER, J.J.C., BAERHEIM-SVENDSEN, A.B.:** Antimicrobial Activities of Essential Oils. A 1976-1986 Literature Review. Aspects of the Test Method; Planta Med. **53**, 395-8 (1987)

Further references on request.

Citation:

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