Impact of Thymol in Thyme Extracts on Their Antispasmodic Action and Ciliary Clearance

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Abstract
Thyme is a herb with broncholytic und secretomotoric effects. Its activity on β2 receptors as a possible mechanism of action was demonstrated. Major components are thymol and carvacrol which are claimed to be responsible for its effects and, therefore, used for standardization in the German pharmacopoeia (0.03% phenols calculated as thymol). Our aim was to investigate the impact of thymol by using thyme extracts with either normal or extremely low thymol concentrations (< 0.005% or > 0.038%). The antispasmodic effect on smooth muscles of the trachea and the ileum and the effect on ciliary activity (respiratory clearance) were investigated. In addition, pure thymol and carvacrol were investigated separately and in spiking experiments. Thymol and carvacrol had a concentration-dependent antispasmodic effect in the rat trachea either being stimulated by acetylcholine, K+ or Ba++. The same result was observed with respect to the increase of mucociliary transport in mice. Extracts with very low thymol contents are effective in all models used except acetylcholine-induced rat ileum contraction. When thyme extracts with normal thymol contents or with very low thymol contents were compared, the extract with normal thymol contents was more effective, both as a relaxant (rat ileum) and as an antispasmodic compound (rat trachea contraction induced by either acetylcholine, Ba++ or K+) and in ciliary transport experiments. Thyme extracts with very low thymol contents (practically free of volatile oil) were equally effective with respect to endothelin effects. When an extract with very low thymol contents is spiked with increasing concentrations of thymol, a concentration-dependent increase concerning the antispasmodic effect (Ba++-induced trachea contraction) is observed. In conclusion, the data show that in various models of antispasmodic effect (ileum and trachea) and by measuring ciliary activity, thymol (and carvacrol) is (are) active, although other not identified components of thyme extract appear to be very important as well, since extracts with very low thymol contents are active. On the basis of these results the standardization on thymol alone appears not to be justified.

Introduction
Obstruction of respiratory airways and damage of the mucociliary transport (MCT) system are part of many known diseases. Secretolytics are used to increase the secretion of mucin from goblet cells; this can be done, e.g., by volatile oils and saponins of plants. Secretomotorics are used to enhance ciliary activity and mucin transport thus accelerating the mucociliary clearance. Volatile oils, saponins and β2-sympathomimetics may modify this effect. Mucolytics (e.g., acetylcysteine [ACC]) depolymerize mucin thus decreasing the viscosity of the mucus layer.

Thymus vulgaris L. (Lamiaceae) is well known as a drug with broncholytic and secretomotoric activity; other thymus species have been investigated as well including Iberian thymes, Thymus serpyllum and other thymes [1–6]. Contractions of the isolated guinea pig trachea, induced by various spasmogens, are antagonized by an ethanolic thyme extract in a reversible and concentration-dependent manner [7]. The same was observed when models of acetylcholine-induced contractions of guinea pig ileum, lung, rat uterus and duodenum were used [8]. The partial involvement of β2 receptors in the effect of thyme on smooth muscle relaxation was demonstrated [9]. The effect of thyme is hypothesized to be dependent,
amongst others, on its volatile oil from which major components such as thymol and carvacrol have been identified [10]. Thymol and carvacrol possess a smooth muscle relaxant effect in a competitive manner at low (10^{-6} M) and in a noncompetitive manner at higher concentrations using rat vas deferens [11]. Thymol relaxes the trachea [12] and its interaction with α1- to α2- and β-receptors of smooth muscles was demonstrated [13]. The action of thymol may be related to a blockade of Na+ channels [14] and an activation of hTRPA1 channels [15]. As an odorant, it may release serotonin via olfactory receptors in the gut [16].

Materials and Methods

Animals

For isolated trachea experiments female Wistar rats weighing 200 to 320 g from a local strain were used (Charles River Laboratories). For studies of the tracheal mucociliary transport function, C57BL/6 mice (Charles River Laboratories) were used. Both mice and rats were allowed food and water ad libitum. All executed studies were approved by the German animal welfare committee.

Compounds/chemicals

Rhodamine 123 fluorescent dye was obtained from Sigma-Aldrich and used at a concentration of 5 × 10^{-5} M for MCT (mucociliary transport) experiments. Barium chloride was from Merck, acetylcholine chloride, endothelin-1 (human/porcine) (purity: > 99%), thymol (purity: > 99.5%) and carvacrol (purity: > 98%) were from Sigma-Aldrich as well. As positive controls we used papaverine (purity 99.8%; Sigma Chemical Co.), salbutamol (purity 97%) from Sigma-Aldrich and bosentan (purity > 99%, kindly provided by Actelion Pharmaceuticals). KCl and all components of the Krebs-Henseleit buffer solution were of analytical grade. Endothelin-1 and bosentan were dissolved in distilled water and then further diluted by Krebs-Henseleit buffer (incubation buffer). Thymol and carvacrol were dissolved in 10% ethanol (stock solution) and further diluted in water.

Plant extracts

The thyme extract was produced according to the pharmacopoeia (Ph. Eur. 5.8). The raw material of thyme (Herba Thymus vulgaris) was collected in Poland shortly before the extraction from cultivation areas under control of the company Martin Bauer and corresponds to the quality of Herba Thymii Pharm. Eur. monograph. A voucher specimen of thyme is kept in Finzelberg's department of quality control under the item no. 2196010. The main constituents of the normal volatile oil in the extract are thymol and carvacrol with contents of 0.15% and 0.015%, respectively. The general relation between thyme herb, thyme oil and thyme extractum fluidum are given in Table 1.

The concentrations of thymol and carvacrol were determined by gas chromatography as an in-process control. The analyzing process was carried out using a Hewlett-Packard 6890 GC system with a flame ionization detector (270°C). Hydrogen was used as the carrier gas (column pressure 0.6 bar, total flow 50 mL/min, split 43.3 mL/min, septum purge 5.3 mL/min) and the column used was a fused silica capillary column, Marcherey & Nagel CW20M (50 m × 0.32 mm; film thickness 0.50 µm). The injector was adjusted to 270°C and the temperature program was initially 160°C for 3 min isothermal and 160°C to 200°C at a rate of 2°C/min, then 200°C for 15 min isothermal. The injection volume was 1 μL thymol (Merck), carvacrol (Merck) and 4-isopropylphenol (Sigma-Aldrich) were used as internal standards. In Fig. 1 gas chromatography fingerprints of the extracts are shown.

Two possibilities exist to produce the thyme extract with high or very low thymol contents. The ethanol fraction (together with the major amount of thymol and carvacrol) in this fluid extract can either be removed by vacuum distillation or not. When this fraction is removed, there is still a very tiny amount of thymol in the extract (< 0.005%, lower than the detection limit). In order to compare the concentrations and extract qualities used in experiments, some data are summarized in Table 2.

A thyme extract (code TPA 178–06; Finzelberg) containing 34.4% ethanol (v/v) was used; the extracting agent was an ethanol/glycerol/ammonia solution (German Pharmacopoeia, 2003). The dry extract content is 11.6%. The production of thyme extracts with very low thymol contents started with the same procedure; ethanol was then removed (TPA 176–06; Finzelberg); 173–06–01 was extracted only by water: dry extract contents of these two extracts are 82.3% and 64.4%, respectively. Concentrations of all extracts used for the experiments were calculated according to their dry substance contents. The final tissue bath concentration of 2 mg/mL TPA 176–06 corresponds to 14.2 mg/mL TPA 178–06 and 2.6 mg/mL TPA 173–06–01. Using the indicated concentration of TPA 178–06, the overall amount of ethanol within the tissue bath was 24.4 μL; this ethanol concentration therefore, was used in control experiments.

Testing in vitro and in vivo

The extracts were tested in vitro (organ bath) at therapeutic and suprapharmacological concentrations. For in vivo experiments, the extract preparations were given intragastrically at a dose of...

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<th>Table 1</th>
<th>Relationship between herb, volatile oil, thymol and carvacrol contents.</th>
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<td>Herb</td>
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<tr>
<td>Thyme herb</td>
<td>Ph. Eur. 6.0</td>
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<td>Thyme oil</td>
<td>Not specified (0.05% volatile oil)</td>
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0.4 and 4.0 mL/kg b.w., correlating with thymol contents of 0.13 and 1.3 mg/kg b.w. and a carvacrol content of 0.015 and 0.152 mg/kg b.w., respectively. The concentration of thyme extracts used for the organ bath experiments and for oral administration was based on their dry powder contents. Since some extracts contained ethanol and since thymol and carvacrol had to be dissolved in ethanol, ethanol controls were included in experiments as necessary.

Contraction experiments (biological experiments)

**Rat trachea:** Wistar rats were killed by diethyl ether narcosis. The whole trachea was dissected and, after removing the adherent tissue, cut into segments of three cartilaginous rings containing tracheal smooth muscle. The endothel was left intact. The tracheal rings were then placed in a 5-mL tissue bath (Mayflower Horizontal Tissue Bath; Hugo Sachs Elektronik) containing pre-warmed (37°C) and gassed (5% CO2, 95% O2) Krebs-Henseleit solution (mM: NaCl 118.1, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25 and glucose 5.6). Tracheal rings were allowed to equilibrate for an hour under a resting tension of 1 cN. The buffer solution was renewed every 15 min. Before starting the experiment, resting tension was set to 0.5 cN and the experiment was begun after a stable baseline was obtained. After addition of an extract/compound and washing, the trachea was allowed to recover until a stable baseline was reached. Tension was increased to 0.5 cN again, if necessary. Tension was recorded isometrically using the force transducer F10 connected to a computer with the HSE ACAD software via the amplifier module TAM 705/1 (all devices and software from Hugo Sachs Elektronik).

Cumulative concentration response curves of ET-1, Ba++ and acetylcholine or K+ using concentrations as indicated in the legends, were performed in the absence and presence of various thyme extracts or thymol, given 10 min (endothelin-1) or 3 min (the other compounds) prior to the compounds that induce contractions. The next higher concentration was added when the contraction of the earlier concentration had reached a plateau, mostly after 10 min.

Contraction by 60 mM K+ and washing of the tracheal segments was repeated until two consecutive contractions were constant. The tested extract/ethanol was added and incubated for 10 min, and then a new peak with 60 mM K+ was induced. Before testing another extract/ethanol a new 60 mM K+ value was measured and normalized to 100% (control). No more than 3 experiments were performed with the same tracheal segment.

**Rat ileum:** The same rats from the trachea experiments were used. The animals were anesthetized and the proximal ileum was removed, washed and placed in Krebs-Henseleit solution. 1-cm segments were placed in 10-mL organ baths with a resting tension of 0.5 g (preload). One end was attached to a force displacement transducer (lever transducer B40, type 373) combined
with a two-channel amplifier (type 301; Hugo Sachs Elektronik); for the recording of tension changes a multi-pen recorder was used (Rikadenki Kogyo). The composition of Krebs-Henseleit solution was (mM): NaCl 118.1, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25 and glucose 5.6. The solution was kept at 37°C, pH 7.4 and gassed with carbogen (95% O2/5% CO2 mixture). Equilibration time for the ileum incubation was 30 min. After the tissues had been pretreated with either the extract or compounds, cumulative concentration-response curves using the agonist acetylcholine chloride were recorded isotonically in the organ bath.

Mucociliary clearance and in vivo microdialysis
Mucociliary clearance was determined as recently described [17]. To measure the mucociliary transport, the mice were anesthetized with Avertin® (0.4 g/kg tribromethanol and 0.4 mL/kg amyl alcohol) and body temperature was maintained at 37°C by a heating pad in combination with a thermo-controller and a temperature probe (CMA Microdialysis). For measurements of the tracheal mucociliary clearance, the trachea was unveiled carefully and a small incision was made directly beyond the larynx. A microcapillary tube (DETKA) with a diameter of 80 µm was loaded with Rhodamine 123 fluorescent dye (15 nL/g body weight) and introduced 16 mm in the trachea. The tube was connected to a microsyringe, so that the dye could easily be placed by pressing 500 nL air into the tube. To prevent a capillary suction effect of the surface of the tube, resulting in immediate detection of the dye, a small silicon sphere (< 100 µm) was placed at the edge of the tube. Leaving the tube in its place, the tip of the microdialysis probe was inserted 4 mm through the same incision into the trachea and fixed in its position by a custom-made retaining jig. It was important not to let air come through the incision by the breathing animal, getting the airways dry and resulting in no measurable transport of the dye. Hence the incision has to be as small as possible, fitting probe and tube. After placing the dye, the mucociliary transport velocity could be calculated from the time the dye needed to travel the defined distance of 12 mm through the trachea, to reach the tip of the microdialysis probe. For all experiments, CMA/20/04 PC probes and a CMA/102 microdialysis pump (CMA Microdialysis) were used. The probe was perfused by phosphate-buffered saline at a constant flow rate of 4 µL/min. After deposition of the fluorescent dye, the dialysate was collected in intervals of 15 s in 96-well Nunc plates with a conical bottom, to take account of the small sample volume of 1 µL per well. The experiment was finished after 24 min, when each of the 96 wells was filled with 1 µL dialysate. The plate was then rapidly inserted into a FluorStar Galaxy fluorescence microplate reader (BMG Lab Tech). The fluorescence intensity recorded as counts in each well was obtained as an equivalent of the dye concentration at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. In earlier test experiments recovery was independent of the dye concentration and it amounted to 10–11% (data not shown). The void volume of the probe with its tubes was obtained by placing the probe directly in a container with rhodamine dye and starting the perfusion immediately. In every performed experiment the dye was detectable in the 5th well (the 5th 15-s time interval), so the time to elute the void volume here was 60 s. This 60 s were subtracted from each calculated dye traveling-time.

In all MCT studies the drugs or saline (control) were administered three times intragastrally (24 h, 12 h and 1 h) before starting the experiment. The data were collected using a standard personal computer with FluorStar Galaxy software (BMG Lab Tech). The first appearance of fluorescent dye in the MCT measurements was detected by averaging the mean background dye concentration and using the first measuring point, which was three SD above this mean background. More details were recently described [9].

Statistical analysis

Differences between two single values were validated by using an unpaired t-test (post hoc test) after having compared all values using ANOVA (GraphPad Prism Version 3.00, GraphPad Software, Inc., 1999). P < 0.05 was regarded as statistically significant, N.S. = not significant.

Results

The direct effects of thymol (and carvacrol) were investigated as these are the lead compounds of the volatile oil of thyme extract. Thymol and carvacrol inhibit K+-induced trachea contractions (Fig. 2). An antispasmodic effect was significant, being 43% at 100 µM thymol (= 15 µg/mL) and 59% at 100 µM carvacrol (= 15 µg/mL).

The effects of thymol and carvacrol on Ba2+-induced trachea contraction were investigated.
Fig. 3 shows the inhibition of the BaCl₂-induced contraction of rat trachea by various concentrations of thymol (left panel) and carvacrol (right panel). Thymol and carvacrol have a concentration-dependent antispasmodic effect. The half-maximal effect (EC₅₀) of thymol is in the range of 70 µg/mL, that of carvacrol in the range of 200 µg/mL. Thymol, therefore, is more potent. These data using Ba++ for contractions are in accordance with data using K⁺ shown in Fig. 2.

In Fig. 4 the effects of thymol und carvacrol on the mucociliary transport (clearance) in the mouse trachea are shown in situ. The animals had been pretreated with the indicated doses of either compound three times. The data show at either dose of pretreatment an increase in mucociliary transport (shortening of the time to overcome the 12-mm distance within the trachea) which indicates an improvement of the ciliary activity. In order to also directly compare thymol and carvacrol, a higher concentration (not present in thyme extracts) of carvacrol was used, i.e., 1.3 mg/kg b. w. The effect of carvacrol is rather the same as a tendency (not significantly different from control) as that of an equivalent dose of thymol.

In the 2nd part of this paper the effects of different thyme extracts with either a normal thymol concentration or with a very low thymol concentration were investigated and compared in several models which should give additional, albeit indirect information on the impact/effectiveness of thymol. Normal thymol concentration means its concentration in the fluid extract represents the concentration of the plant, in our investigation 0.038%.

For testing the antispasmodic effects, both ileum and trachea were used with various stimulating compounds such as acetylcholine, BaCl₂, KCl and endothelin. Fig. 5a shows the effect of both a thyme extract with normal thymol contents and with very low thymol contents on acetylcholine-induced rat ileum contraction. In contrast to the thyme extract with normal thymol, the thyme extract with a very low thymol content did not show an antispasmodic effect. It may be speculated that thymol-antagonistic effects in the thyme extract with low thymol lead to some contraction which may be the result of compounds interacting with β₂ receptors not present in ileum.

In a similar type of experiment for measuring the antispasmodic effect, the smooth muscle of trachea instead of ileum and BaCl₂ instead of acetylcholine as a stimulant were used. Ba++ acts as a depolarizing and, therefore, contracting compound. Both the

Table 3 Direct effect of different thyme extracts using the smooth muscle of rat ileum. Mean ± SEM, n = 8 independent experiments. Data are normalized to 100% which is the effect of contraction induced by 10 µM acetylcholine.

| Thyme extract with very low thymol contents (<0.005%) | 0.24 ± 1.37 |
| Thyme extract with normal thymol contents (>0.038%) | -17.07 ± 8.47 (N.S.) |

* P < 0.05 vs. control.
thyme extracts with normal thymol and low thymol contents had an effect, although the extract with low thymol was less effective (Fig. 5b).

In Fig. 6 the result of testing the antispasmodic effect of various extracts using K⁺ as a spasmogen (depolarizing compound) is shown. All three extracts decreased tracheal contraction induced by 60 mM K⁺ compared to the control (Fig. 6). The ethanol extract TPA 178–06 (normal thymol) had a stronger influence than the ethanol-free extracts TPA 176–06 and TPA 173–06–1 (low thymol). Ethanol itself did not alter the contraction (106 ± 5; N.S.). It is obvious that both extracts with low contents of thymol influence the tracheal contraction.

Next the antispasmodic effect of different thyme extracts on the endothelin-induced rat trachea contraction was investigated. Both thyme extracts (5 mg/mL) inhibit the endothelin-1 induced trachea contraction to a similar extent (Fig. 7). In contrast to bosentan (competitive leftward shift of the endothelin-1 curve) the effects of the thyme extracts were noncompetitive.

Thyme extracts increased the mucociliary transport (shortened time for the distance) (Fig. 8); the extract containing low thymol was weaker.

Since an extract with very low thymol is effective in attenuating smooth muscle contraction (shown in Fig. 5b), indicating that thymol as the major effective principle is not necessary although...
Thymol and carvacrol are effective since they possess a concentration-dependent antispasmodic effect in smooth muscles such as rat ileum and trachea independent of the type of stimulation (acetylcholine, K⁺ or Ba²⁺). However, it has to be noted that the concentrations used are higher than those usually present in thyme extracts in order to obtain pharmacological concentration response data. A thyme extract with low thymol contents reduces contractions by ~40% when Ba²⁺ or endothelin-1 are used as stimulants, indicating that in this extract thymol is not the compound of major importance.

Thymol and carvacrol are effective with respect to the increase of muco-ciliary transport in the mouse. These data were obtained using reasonable doses in mice.

When 14.2 mg/mL thyme extract with normal thymol contents (TPA 178–06 and with 0.038% thymol) was used, which is equivalent to 35.9 µM thymol and 5.396 µg/mL thymol, the Ba²⁺-induced trachea contraction is reduced by >30% (Fig. 5b). Addition of a rather low thymol concentration (1.91 µg/mL in Fig. 9) does not show any additional effect: this may be interpreted in that thymol is not that important, at least not in this model.

The question is relevant whether these used thymol concentrations are relevant. From preparation dosages the concentrations are about 10 µg/mL body fluid which is 1000 times lower than that used in our experiments; the concentrations at the trachea, however, are probably much higher when thymol with the volatile oil is exhaled.

When thyme extracts with normal thymol contents (>0.038%) or with low thymol contents (<0.005%) were compared, the extract with normal thymol contents was more effective as a relaxant in rat ileum (cholinergic stimulation). These data are interpreted in that thymol is important for anticholinergic effects and appears to be involved in neurotropic-spasmolytic effects. Thymol, however, is not important for the overall antispasmodic effect when all other spasmod triggers are considered, e.g., when a general depolarization induces the spasm: e.g., Ba²⁺ and K⁺, effect on trachea. In experiments using endothelin stimulation there was no major difference: both extracts were active which means thymol is not important. Thymol alone had no effect on endothelin-induced trachea contraction as was recently shown [20].

Based on the experimental data shown above, extracts with low thymol contents are sufficiently active except with respect to ileum smooth muscle. When an extract with very low thymol contents is spiked with increasing concentrations of thymol, there was a concentration-dependent increase in antispasmodic effect (Ba²⁺-induced contraction) only at normal levels. Corroborating other publications, the thymol fraction (phenols) is not as essential for the effect due to their low concentrations [19].

In conclusion: altogether it is clear that thymol and carvacrol are important compounds of thyme extract; thymol contributes to the antispasmodic effect in most models. The data show that extracts with low thymol are active: in most experiments it increases the relaxation of smooth muscle. Thymol (and carvacrol) are active although other not identified components of thyme extract appear to be important as well. Further standardization on thymol (phenols) alone needs to be reconsidered.

References

10 Kommission E of BfArM, Germany
15 Lee SP, Buber MT, Yang Q, Cerne R, Cortés KY, Sprous DG, Bryant RW. Thymol and related alkyl phenols activate the hTRPA1 channel. Br J Pharmacol 2008; 153: 1–11
18 Van Den Broucke CO, Lemli JL. Spasmolytic activity of the flavonoids from Thymus vulgaris. Pharm Weekbl Sci 1983; 5: 9–14