

1                   Susceptibility of Drug-Resistant Clinical HSV-1 Strains  
2                   to Essential Oils of Ginger, Thyme, Hyssop and Sandalwood

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22 **ABSTRACT**

23 Acyclovir-resistant clinical isolates of herpes simplex virus type 1 (HSV-1) were  
24 analysed *in vitro* for their susceptibility to essential oils of ginger, thyme, hyssop and  
25 sandalwood. All essential oils exhibited high levels of virucidal activity against acyclovir-  
26 sensitive strain KOS and acyclovir-resistant HSV-1 clinical isolates and reduced plaque  
27 formation significantly.

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42 Key words: essential oil, virucidal activity, herpes simplex virus, acyclovir-resistant HSV-1

43 running title: Essential oils affect drug-resistant HSV

44 Herpes simplex virus type 1 (HSV-1) is a highly prevalent pathogen among children  
45 and adults causing primary infections which present clinically as herpes labialis or as primary  
46 herpetic gingivostomatitis and is able to establish a latent infection in the nervous system that  
47 can be reactivated quite frequently (10, 31, 32). Acyclovir has been widely used for the  
48 management of herpes virus infections, its preferential phosphorylation by the HSV-encoded  
49 thymidine kinase (TK) makes it a selective antiviral drug (8, 14). The emergence of virus  
50 strains resistant to commonly used anti-herpesvirus drugs is a problem in the clinical setting,  
51 particularly in immunocompromised patients (3, 4, 6, 19, 30). This trend has led to search for  
52 alternative antiherpetic agents that have a wide range of efficacy without serious adverse  
53 effects, and which are effective for viral strains resistant to current antiviral agents. HSV  
54 develops resistance predominantly as a result of mutations in genes that code for thymidine  
55 kinase (TK), but resistance can also result from mutations in DNA polymerase. The  
56 antiherpes activity of Australian tee tree oil (16, 23), peppermint oil (25) and manuka oil (17)  
57 has previously been published. In the current study we analysed the virucidal activity of  
58 essential oils derived from ginger, thyme, hyssop and sandalwood against acyclovir-sensitive  
59 and acyclovir-resistant clinical HSV-1 isolates in which therapy with acyclovir failed.

61 Essential oils from ginger (*Zingiber officinale*), thyme (*Thymus vulgaris*), hyssop  
62 (*Hyssopus officinalis*) and sandalwood (*Santalum album*) were investigated. The main  
63 components comprising about 5 – 10% in ethanolic extracts of ginger oil are sesquiterpenes,  
64 e.g. zingiberene,  $\beta$ -bisabelene, sesquiphellandrene and curcumen; thyme oil consists mainly  
65 of thymol and carvacrol, hyssop oil consists mainly of monoterpenes, e.g. 1-pinocamphone,  
66 isopinocamphone, pinocarvone and  $\alpha$ -pinene; sandalwood oil is mainly composed of  
67 sesquiterpenealcohols, e.g. santalol, bergamotol and santalene. Acyclovir-sensitive herpes  
68 simplex virus type 1 (HSV-1) strain KOS (15) and acyclovir-resistant patient isolates 1246/99

69 and 496/02 were used for experiments. Both hospital specimens from infected patients  
70 revealed a single point mutation in the coding sequence of the TK gene which result in  
71 frameshifts and probably only truncated nonfunctional thymidine kinase is expressed. These  
72 mutations were both located in homopolymer stretches of guanines downstream of the ATP-  
73 binding site for 1246/99 and cytosines downstream of the nucleoside-binding site for 496/02  
74 and have been reported previously (1, 5, 9, 21, 22). The well characterized acyclovir-resistant  
75 HSV-1 strain Angelotti was also used in the experiments and exhibits a single-point mutation  
76 in the DNA polymerase gene (12). Viruses were routinely grown on RC-37 cells as described  
77 previously (20). Genomic DNA was extracted from the supernatant of plaque purified virus  
78 and amplified by PCR (5) and PCR products were sequenced as described previously (24).  
79 All essential oils were dissolved in ethanol and added to the medium at a final concentration  
80 of 1% ethanol for cytotoxicity assays which determines the viability and proliferation of the  
81 cells (25, 29).

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83 The cytotoxic concentration of the drug which reduced viable cell number by 50%  
84 ( $CC_{50}$ ) and the effective concentration of the test compound which inhibited plaque numbers  
85 by 50 % ( $EC_{50}$ ) was determined from dose-response curves (Table 1). Selectivity indices for  
86 different essential oils were calculated as the ratio  $CC_{50}/EC_{50}$  and are given in Table 1. Ginger  
87 oil and hyssop oil exhibited selectivity indices of 20 and 75, respectively. The maximum  
88 noncytotoxic concentration of the tested essential oils was determined at 0.003% for ginger  
89 oil, 0,005% for thyme oil and hyssop oil and 0.0006% for sandalwood oil. The dose-response  
90 curves are shown in Fig. 1 demonstrating a dose-dependent activity of the tested essential oils.  
91 The inhibitory effect of the essential oils against HSV was tested by adding the oils at  
92 different times during the infection cycle of HSV (Table 2). To identify the step at which  
93 replication might be inhibited, cells were infected with these HSV-1 strains after

94 preincubation of the cells for 1 hour with essential oils, pretreatment of the virus strains for 1  
95 hour with the essential oils prior to infection, addition of the essential oils during adsorption  
96 or after adsorption during the intracellular replication period. In all experiments untreated  
97 virus infected cells were used as control. The percent reduction was calculated relative to the  
98 amount of virus produced in the absence of the compounds. Pretreatment of HSV with the  
99 analysed essential oils prior to infection caused a significant reduction of infectivity ranging  
100 from 95.9% to 99.9% for acyclovir-sensitive and drug-resistant HSV-1 strains.

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102 These results indicate that essential oils derived from ginger, thyme, hyssop and  
103 sandalwood affected the virus before adsorption and in a different manner than acyclovir  
104 since plaque formation of acyclovir-resistant patient isolates HSV-1 1246/99 and 496/02 was  
105 significantly reduced, too. A high level of virucidal activity during the pretreatment of HSV-1  
106 by using the essential of *Salvia fruticosa* was detected previously (28). Essential oils seem to  
107 be mostly efficient on cell-free virus but with limited effect on virus replication in cells and  
108 on cell to cell spread of the virus (13). These results suggest that the investigated essential oils  
109 might interfere with virion envelope structures which are necessary for adsorption or entry  
110 into host cells or might dissolve the HSV envelope. Treatment of HSV-1 with oregano  
111 essential oil has been shown to disrupt the viral envelope (27). In preliminary  
112 electronmicroscopical studies we could also demonstrate a disruption of the viral envelope  
113 after pretreatment of HSV with essential oils thereby impairing their ability to infect host cells.  
114 Shogan et al. (26) investigated the antiviral mechanisms of a GT-rich oligonucleotide which  
115 potentially inhibited attachment of herpes simplex virus to cells by induction of a  
116 conformational change in glycoprotein B, resulting in inactivation of infectivity. The virucidal  
117 activity of the GT-rich oligonucleotide is time-dependent and causes an irreversible loss of  
118 infectivity. A resistant mutant virus with mutations in the UL27 gene could be isolated by

119 these authors and attachment of HSV to cells was no more inhibited in the mutant strain.  
120 Since lipophilic essential oils inhibit attachment only moderately and most likely exert their  
121 virucidal activity by disrupting the viral lipid membrane, resistant strains of HSV could not be  
122 detected. After pre-treatment of HSV with essential oils, the few remaining infectious viruses  
123 are still sensitive to treatment with essential oils. Essential oils are complex mixtures of  
124 compounds with low molecular weight, such as monoterpene hydrocarbons, sesquiterpene  
125 hydrocarbons, their corresponding oxidized products, e.g. alcohols, aldehydes and ketones, as  
126 well as homologues of phenylpropanoids and small amounts of diterpenoids. The active  
127 components of essential oils might consist of lipophilic carbohydrates that interact with the  
128 lipid membrane (18). These active substances in antibacterial activity (7, 11) might exhibit a  
129 similar activity against viral envelopes. Interestingly acyclovir-resistant clinical isolates were  
130 significantly inhibited by the essential oils, the titre of HSV was reduced by 95.9% to 99.9%.  
131 Since essential oils are able to inhibit acyclovir-resistant HSV-1 isolates, the mechanism of  
132 interaction between these compounds and acyclovir with HSV must be different. Acyclovir  
133 inhibits virus replication by interference with the DNA polymerase inside the cell, whereas  
134 essential oils probably inactivate HSV before it enters the cell. The effective dosage for a  
135 systemic application of essential oils is rather high and leads to cytotoxic effects.  
136 Furthermore, a short term systemic bioavailability makes a systemic application unlikely.  
137 Therefore other antiherpetic agents which are effective against viral mutants resistant to  
138 current antiviral agents are of great interest as additional topical treatment in recurrent  
139 acyclovir-susceptible and acyclovir-resistant HSV-1 infections as has been demonstrated by  
140 topical application of tea tree oil (2) against recurrent herpes labialis.

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TABLE 1. Selectivity indices (SI) of essential oils for HSV-1

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essential oil	CC <sub>50</sub> (%)	EC <sub>50</sub> (%)	selectivity index (SI)
ginger oil	0.004 ± 0.001	0.0002 ± 0.00001	20
thyme oil	0.007 ± 0.0003	0.001 ± 0.0001	7
hyssop oil	0.0075 ± 0.002	0.0001 ± 0.00001	75
sandalwood oil	0.0015 ± 0.0001	0.0002 ± 0.000003	7

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Experiments were repeated independently two times and data presented are the mean of three experiments.

305 TABLE 2. Virucidal effect of essential oils against acyclovir-sensitive HSV-1 strain KOS and acyclovir-resistant strain Angelotti and acyclovir-  
 306 resistant clinical isolates 1246/99 and 496/02

	ginger oil				thyme oil				hyssop oil				sandalwood oil			
	KOS	Ang.	1246/99	496/02	KOS	Ang.	1246/99	496/02	KOS	Ang.	1246/99	496/02	KOS	Ang.	1246/99	496/02
311 pretreatment cells	101.3	99.4	109.3	87.9	101.1	93.9	117.9	89.3	95.7	89.2	104.8	98.6	104.3	97.1	104.7	85.9
312	±8.4	±5.5	±3.2	±4.1	±8.0	±18.1	±10.4	±1.0	±10.7	±6.4	±4.8	±2.3	±2.6	±8.5	±7.7	±1.6
313 pretreatment virus	0.1	0.2	0.3	0.1	3.4	4.1	1.2	0.3	0.1	0.2	0.3	0.1	1.1	0.2	1.1	0.3
314	±0.1	±0.1	±0.2	±0.1	±2.9	±3.2	±0.9	±0.3	±0.1	±0.2	±0.3	±0.1	±1.9	±0.2	±0.8	±0.2
315 adsorption	66.5	70.7	68.2	47.1	92.2	91.3	87.2	61.1	76.1	105.3	80.0	51.2	104.3	89.8	71.0	
316	±4.0	±3.1	±9.4	±0.9	±9.2	±5.0	±8.3	±3.3	±5.9	±12.0	±10.0	±4.9	±2.9	±8.2	±5.9	±5.2
317 replication	87.2	101.6	95.4	100.8	101.5	99.3	99.1	98.4	88.1	97.6	106.8	99.7	87.4	85.0	105.5	93.6
318	±19.5	±8.3	±6.0	±3.3	±15.9	±0.5	±11.3	±0.8	±10.2	±7.0	±10.0	±4.0	±18.9	±0.5	±2.6	±0.1

325 Maximum noncytotoxic concentration of the essential oils were used for all experiments. Data represent percentages of plaques  
 326 compared to untreated controls. Experiments were repeated independently two times and data presented are the mean of three experiments.

327 **FIGURE LEGENDS**

328

329 **Fig. 1.** Determination of the 50% inhibitory concentration ( $EC_{50}$ ) of ginger oil, thyme  
330 oil, hyssop oil and sandalwood oil against HSV-1. Viruses were incubated for 1 hour at room  
331 temperature with increasing concentrations of the essential oils and immediately tested in a  
332 plaque reduction assay. Experiments were repeated independently two times and data  
333 presented are the mean of three experiments.

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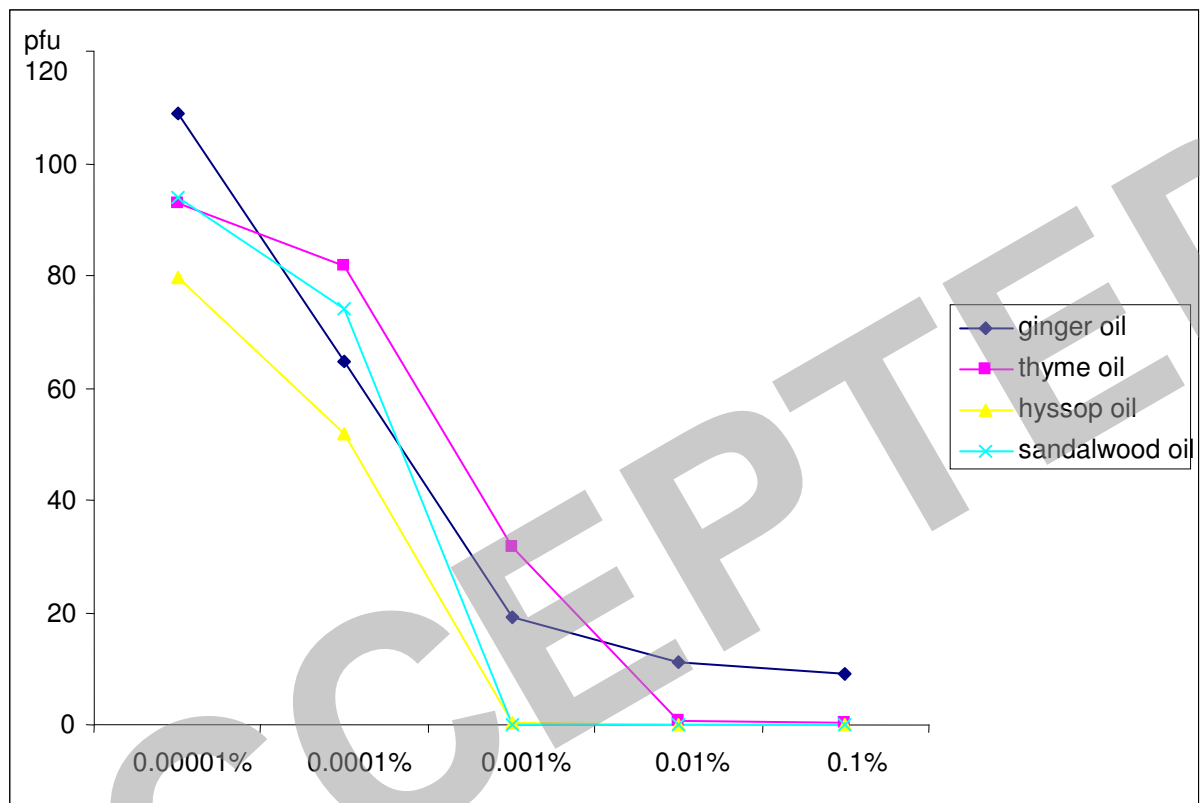
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352 FIG. 1

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