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In Vitro Acaricidal Activity of the Thymol against *Sarcoptes scabiei* and Regulating Effects on Enzyme Activity

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Abstract

Background: Thymol found in many essential oils of natural plants is a monoterpene. We had confirmed that thymol is one of the main components of *Elsholtzia densa* Benth essential oil and the essential oil showed significant effects against *Sarcoptes scabiei in vitro* in our laboratory. In this paper, the acaricidal activity and the mechanism of the thymol against *Sarcoptes scabiei* were investigated based on enzyme activity.

Results: The data of toxicity tests were analyzed by a complementary log-log (CLL) model. The results showed that thymol had significant effects against mites, 8 and 16 mg/ml of thymol had strong toxicity against *Sarcoptes scabiei*, with LT_{50} values of 1.406 and 0.825 h. The LC_{50} values were 3.829 mg/ml for *S. scabiei* in 4 h. The activity of Superoxide dismutase (SOD), peroxidase (POD), monoamine oxidase (MAO), glutathione-s-transferases (GSTs) and Ca^{2+}-ATPase are significantly changed after treatment with thymol compared with the control group. SOD, POD, MAO and Ca^{2+}-ATPase in treated mites were suppressed in activity, whereas that of GSTs was activated.

Conclusion: The mechanism of the acaricidal activity of the thymol was mainly achieved through interference with the energy metabolism and nerve conduction of the mites, thus leading to the mite death.

Keywords: Acaricidal activity; Thymol; *Sarcoptes scabiei*; Enzyme activity

Abbreviations

E. densa: *Elsholtzia densa*; LC_{50}: Median lethal concentration; LT_{50}: Median lethal time; *S. scabiei*: *Sarcoptes scabiei*; SOD: Superoxide dismutase; POD: Peroxidase; MAO: Monoamine oxidase; GSTs: Glutathione-s-transferases; CLL: Complementary log-log.

Introduction

Animal acariasis such as *Sarcoptes scabiei*, can cause very important veterinary skin diseases [1]. This infection can reduce the production and the quality of animal products and even lead to death in large infestation [2,3]. Because of its highly contagious, it is more difficult to treat than other parasitic disease [4].

Nowadays, many chemical drugs have been widely used to treat and control sarcoptic mange in the clinic, such as ivermectin [5], amitraz [6] and abamectin [7]. These drugs could bring some good treatment outcomes, however, chemical synthetic drugs have also caused the environmental pollution, drug residue, and abamectin have led to the multiple resistances of the target species [8].

Thymol, a monoterpene is first found from *Thymus mongolicus* Ronn [9], subsequently found in other plants like *Origanum vulgare L* [10] and *Trachyspermum ammi Sprague* [11]. A large number of reports have indicated that thymol has a very wide biologic activity, such as antimicrobial activities [12-14] and acaricide activity against engorged females and unengorged larvae of *Rhipicephalus microplus* [15], nimphs and engorged larvae of *Rhipicephalus sanguineus* [16] and *Amblyomma cajennense* [17].

Studies have shown that thymol can reduce environmental contamination because of its rapid dissipation and low level of residues [18,19]. In USA, thymol is approved as a food additive that is safe to human health by FDA [20]. We previously showed that the essential oil from *Elsholtzia densa* Benth had significant effects against *Sarcoptes scabiei in vitro* and thymol is one of the main components of the essential oil in our laboratory, so the aim of the work was to evaluate the acaricidal activity of thymol against *sarcoptes scabiei* directly and explain the acaricidal mechanism based on the enzyme activities for the purpose of exploiting new acaricide.

Materials and Methods

Chemicals

The study was developed at the Thymol and ivermectin were supplied by Shanghai Zhanyun Chemical Co., Ltd. (Chengdu. China). Superoxide dismutase (SOD), Ca^{2+}-ATPase reagents, peroxidase (POD), monoamine oxidase (MAO) and glutathione-s-transferases (GSTs) were purchased from Nanjing Jiancheng Bioengineering Institute. Due to their low water solubility, thymol solutions were mixed under heating (40) in paraffin liquid.

Collection of mites

The rabbits that were naturally infected with *Sarcoptes scabiei* were obtained from a farm affected by an outbreak. All rabbits had not been treated with any anti-ascariasis drug before mites were collected. The collected scabs were placed in Petri dishes and then incubated immediately at 35°C for 30 min in an incubator. The motile adult mites were immediately collected for testing under a stereomicroscope. Under an optical microscope, adults have eight legs, which make them easily recognizable from larvae which have six legs. After collection of the samples, all the infected rabbits were treated with ivermectin immediately. Sampling exercise adhered to ethical and animal care guidelines and all processes were conducted in accordance with the Guide for the Care and Use of Laboratory Animal [21].

Acaricidal activity *in vitro*

Due to their low water solubility, thymol solutions were mixed under heating (40℃) in paraffin liquid for obtaining five concentrations: 1, 2, 4, 8, 16 mg/ml. The 0.1 ml sample was directly added to the small petri dishes (5 cm in diameter, 2 cm deep) and the mites were placed in it with 10 specimens per dish. 1% of the ivermectin was used as a positive control and liquid paraffin as a negative control. All experiments were performed in 6 replicates. All dishes were incubated at 25℃ under 75% relatively humid [22] and were observed under a stereomicroscope at 1, 2, 4, 8, 16 and 24 h. Mites were considered to have died when they lack of reaction by stimulating with a needle.

Determination of enzyme activities

According to previously described method [23], The 10 μl thymol solutions with concentrations of 10 mg/ml, 5 mg/ml, and 2.5 mg/ml were added to plastic petri dishes and paraffin liquid was added in a control group. The mites were placed in small dishes with 200 mites per dish. All dishes were incubated at 25℃ and 75% relative humidity [22]. After culturing for 2, 4, 8, 16, 24 hours the mites of each group were placed in a homogenizer immediately. They were homogenized with 0.3 ml normal saline in cool water. Next the mites in the homogenates were transferred to a centrifuge tube. After centrifugation at 2500 × g for 10 min under 4℃, the supernatant was collected as the enzyme extract and used to test the activities of Superoxide dismutase (SOD), Ca^{2+}-ATPase reagents, peroxidase (POD), and glutathione-s-transferases (GSTs). Three repeats for each group.

Data analysis

The obtained data was analyzed with the statistical software (SPSS, version 20.0) and are expressed as the means ± SDs. The median lethal concentration value (LC 50) and the median lethal time value (LT 50) were calculated by the complementary log-log (CLL) model [21].

Results

Acaricidal activity *in vitro*

We studied the acaricidal activity of thymol against *sarcoptes scabiei in vitro*. The results showed that treatment with a high concentration group (16 mg/ml thymol) caused 58.33%, 65.00%, 76.67%, 86.67%, 93.33%, 100% mortality in test mites at 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, respectively (Table 1). A high concentration group (16 mg/ml of thymol) caused 100% mortality in test mites (Figure 1). And most mites remained alive after 24 hours treatment period in the Negative control treatment (paraffin liquid only). However, in the positive control group of ivermectin, some mites still remained alive in the sample after 24 hours. The reason why they were alive probably is that the mites produced resistance to the ivermectin. Compared to the control group, the thymol showed significant effects against *Sarcoptes scabiei in vitro*. The toxicity of the thymol was evaluated using a CLL model, Pearson's Chi-square test and the Hosmer-Lemeshow goodness-of-fit statistic indicated that the date fitted the CLL model. The LC 50 and LT 50 values of thymol against *Sarcoptes scabiei* are shown in Tables 2 and 3 respectively.

Concentration (mg/mL)	Time (h)					
	1 h Mean Mortality (%) ± SD	2 h Mean Mortality (%) ± SD	4 h Mean Mortality (%) ± SD	8 h Mean Mortality (%) ± SD	16 h Mean Mortality (%) ± SD	24 h Mean Mortality (%) ± SD
1 mg/mL	11.67 ± 4.00^{Df}	20.00 ± 6.33^{Cd}	28.33 ± 7.53^{De}	31.67 ± 7.53^{Ee}	40.00 ± 6.00^{De}	45.00 ± 5.48^{De}
2 mg/mL	20.00 ± 6.33^{CDe}	31.67 ± 7.53^{Bc}	40.00 ± 6.33^{CDd}	50.00 ± 6.33^{Dd}	56.67 ± 5.10^{Cd}	65.00 ± 5.48^{Cd}
4 mg/mL	28.33 ± 4.08^{BCd}	41.67 ± 7.53^{Bb}	51.67 ± 7.53^{BCc}	60.00 ± 6.33^{Cc}	65.00 ± 8.37^{Cc}	76.67 ± 8.17^{Bc}
8 mg/mL	50.00 ± 6.33^{Ab}	58.33 ± 7.53^{Aa}	56.67 ± 8.17^{Bbc}	68.33 ± 7.53^{BbC}	81.67 ± 4.08^{Bb}	93.33 ± 5.16^{Ab}
16 mg/mL	58.33 ± 7.53^{Aa}	65.00 ± 8.37^{Aa}	76.67 ± 10.33^{Aa}	86.67 ± 5.16^{Aa}	93.33 ± 8.17^{Aa}	100.00 ± 0.00^{Aa}
Positive control	38.33 ± 13.29^{Bc}	40.00 ± 8.94^{Bbc}	65.00 ± 13.78^{ABb}	75.00 ± 5.48^{Bb}	80.00 ± 6.33^{Bb}	81.67 ± 7.53^{Bc}
Negative control	0.00 ± 0.00^{Eg}	0.00 ± 0.00^{De}	0.00 ± 0.00^{Ef}	1.67 ± 4.00^{Ff}	0.00 ± 0.00^{Ef}	0.00 ± 0.00^{Ef}

Table 1: The acaricidal activity of the thymol against *Sarcoptes scabiei in vitro*. The difference between data with the different capital letter within a column is extremely significant (P<0.01), and the difference between data with the different small letters within a column is significant (P<0.05).

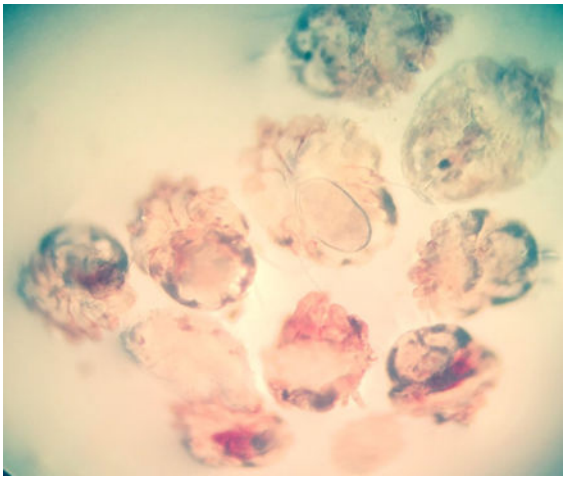

Figure 1: Dead *Sarcoptes scabiei* observed by microscopy following treatment with thymol after 24 hours.

Concentration	Regression line	LT_{50} (h) (95% FL)	Pearson chi-square
1 mg/mL	y=0.712 × -1.094	34.518 (18.315-134.185)	6.821
2 mg/mL	y=0.798 × -0.706	7.662 (5.211-12.273)	5.838
4 mg/mL	y=0.846 × -0.515	4.060 (12.640-5.853)	7.918
8 mg/mL	y=0.880 × -0.130	1.406 (0.072-2.182)	13.498
16 mg/mL	Y=1.191 × +0.100	0.825 (0.406-1.267)	16.985

Table 2: The probit regression analysis of toxicity (LT_{50}) of the thymol against mites *in vitro*.

Time (h)	Regression line	LC 50 (mg/mL) (95%FL)	Pearson chi-square
1 h	y=1.218 × -1.212	9.896 (7.193-15.936)	5.323
2 h	y=1.042 × -0.812	6.023 (4.345-9.163)	6.863
4 h	y=0.994 × -0.579	3.829 (2.649-5.477)	8.805
8 h	y=1.187 × -0.447	2.383 (1.623-3.195)	6.878
16 h	y=1.356 × -0.290	1.635 (1.079-2.184)	9.011
24 h	y=1.875 × -0.195	1.271 (0.898-1.623)	8.494

Table 3: The probit regression analysis of toxicity (LC_{50}) of the thymol against mites *in vitro*.

Enzyme Activity

Superoxide dismutase (SOD) activity

From the Table 4, we can see that the changes in the SOD activity of the mites treated with thymol showed wave-like appearance similar to that of the control group. However, the SOD activity of rabbit scabies mites was significantly lower than the control group ($P<0.05$) during the 24 hours after treatment. At the same time point, the high-dose group exhibited a larger decrease than the low-dose group. The inhibition rate of SOD activity was 51.81%, 38.41%, 23.82% as compared to the control group in the concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml after 24 h. The results showed that the higher the drug concentration, the more the SOD activity was inhibited.

Groups	SOD activity of the different time points after drug treatment (U/mg prot)				
	2 h	4 h	8 h	16 h	24 h
10 mg/mL	17.12 ± 0.11^{Dd}	21.69 ± 0.39^{Dd}	17.25 ± 0.15^{Dd}	16.46 ± 0.13^{Dd}	8.91 ± 0.13^{Dd}
5 mg/mL	22.42 ± 0.18^{Cc}	25.81 ± 0.16^{Cc}	23.52 ± 0.13^{Cc}	20.87 ± 0.08^{Cc}	11.45 ± 0.18^{Cc}
2.5 mg/mL	27.53 ± 0.20^{Bb}	29.05 ± 0.08^{Bb}	28.53 ± 0.46^{Bb}	26.15 ± 0.12^{Bb}	17.46 ± 0.12^{Bb}
Paraffin liquid	34.18 ± 0.24^{Aa}	38.33 ± 0.15^{Aa}	36.04 ± 0.15^{Aa}	33.21 ± 0.68^{Aa}	27.24 ± 0.68^{Aa}

Table 4: Effects of thymol on the activity of SOD against *Sarcoptes scabiei in vitro*. The difference between data with the different capital letter within a column is extremely significant ($P<0.01$), and the difference between data with the different small letters within a column is significant ($P<0.05$).

Peroxidase (POD) activity

The treated mites had lower POD activity than that the control mites during the 24 h after treatment. The POD activity of mites treated for 24 h was significantly suppressed compared with the control group ($P<0.05$). The changes in the POD activity of mites treated with thymol are shown in Table 5. In the low-and middle-does experimental groups, the change of the POD activity follows a trend of decline, but fluctuated in the high-dose experiment group.

Groups	POD activity of the different time points after drug treatment (U/mg prot)				
	2 h	4 h	8 h	16 h	25 h
10 mg/mL	0.019 ± 0.002^{Dd}	0.028 ± 0.002^{Dd}	0.012 ± 0.001^{Dd}	0.011 ± 0.001^{Dd}	0.009 ± 0.002^{Dd}
5 mg/mL	0.023 ± 0.002^{Cc}	0.021 ± 0.002^{Cc}	0.017 ± 0.001^{Cc}	0.017 ± 0.002^{Cc}	0.013 ± 0.001^{Cc}
2.5 mg/mL	0.041 ± 0.003^{Bb}	0.041 ± 0.001^{Bb}	0.039 ± 0.002^{Bb}	0.028 ± 0.001^{Bb}	0.025 ± 0.001^{Bb}
Paraffin liquid	0.085 ± 0.001^{Aa}	0.085 ± 0.001^{Aa}	0.086 ± 0.001^{Aa}	0.086 ± 0.001^{Aa}	0.086 ± 0.001^{Aa}

Table 5: Effects of thymol on the activity of POD against *Sarcoptes scabiei in vitro*. The difference between data with the different capital

letter within a column is extremely significant (P<0.01), and the difference between data with the different small letters within a column is significant (P<0.05).

Monoamine oxidase (MAO) activity

The changes in MAO activity of the rabbit scabies mites treated with thymol are shown in Table 6. The results showed that thymol obviously inhibit the MAO activity in the mites. The MAO activity of the mites in the treatment groups was significantly lower than the control group (P<0.01).

Groups	MAO activity of the different time points after drug treatment (U/mg prot)				
	2 h	4 h	8 h	16 h	24 h
10 mg/mL	12.43 ± 0.26^{Cd}	9.33 ± 0.30^{Dd}	7.34 ± 0.25^{Dd}	5.36 ± 0.31^{Dd}	3.47 ± 0.17^{Dd}
5 mg/mL	16.74 ± 0.27^{Bc}	12.19 ± 0.39^{Cc}	9.64 ± 0.23^{Cc}	8.22 ± 0.19^{Cc}	7.31 ± 0.25^{Cc}
2.5 mg/mL	17.41 ± 0.34^{Bb}	15.21 ± 0.25^{Bb}	12.53 ± 0.18^{Bb}	11.23 ± 0.18^{Bb}	9.51 ± 0.30^{Bb}
Paraffin liquid	20.00 ± 0.34^{Aa}	19.77 ± 0.16^{Aa}	18.63 ± 0.27^{Aa}	18.15 ± 0.22^{Aa}	16.61 ± 0.41^{Aa}

Table 6: Effects of thymol on the activity of MAO against *Sarcoptes scabiei in vitro*. The difference between data with the different capital letter within a column is extremely significant (P<0.01), and the difference between data with the different small letters within a column is significant (P<0.05).

Glutathione-s-transferases (GSTs) activity

Compared with the control group (Table 7), the GST activity of thymol-treated group was activated within 24 h. The GST activity of control group had no noticeable change after treatment for 24 h. The GST activity of thymol-treated group was first decrease then increase then decrease, but showed no remarkable change after 16 h.

Groups	GST activity of the different time points after drug treatment (U/mg prot)				
	2 h	4 h	8 h	16 h	24 h
10 mg/mL	3.389 ± 0.035^{Aa}	2.611 ± 0.069^{Aa}	3.531 ± 0.024^{Aa}	2.179 ± 0.043^{Aa}	1.904 ± 0.039^{Aa}
5 mg/mL	3.350 ± 0.047^{Aa}	2.520 ± 0.042^{Ab}	3.361 ± 0.045^{Bb}	1.852 ± 0.048^{Aab}	1.910 ± 0.033^{Aa}
2.5 mg/mL	2.344 ± 0.055^{Bb}	2.305 ± 0.033^{Bc}	3.264 ± 0.046^{Bc}	1.738 ± 0.619^{Aab}	1.808 ± 0.025^{Bb}
Paraffin liquid	1.506 ± 0.022^{Cc}	1.499 ± 0.012^{Cd}	1.488 ± 0.016^{Cd}	1.488 ± 0.016^{Bc}	1.514 ± 0.013^{Cc}

Table 7: Effects of thymol on the activity of GST against *Sarcoptes scabiei in vitro*. The difference between data with the different capital letter within a column is extremely significant (P<0.01), and the difference between data with the different small letters within a column is significant (P<0.05).

Ca^{2+}-ATPase activity

The changes in the Ca^{2+}-ATPase activity of mites treated with thymol are shown in Table 8. Compared with the control group, the Ca^{2+}-ATPase activity of mites in the treatment groups decreased significantly (P<0.01), and declined with rise of thymol concentration. Over time, the Ca^{2+}-ATPase activity of control group increased and then decreased within 24 h, and the activity of treatment group had a descending trend except the treatment group at concentration of 10 mg/ml.

Groups	Ca^{2+}-ATP activity of the different time points after drug treatment (U/mg prot)				
	2 h	4 h	8 h	16 h	24 h
10 mg/mL	1.220 ± 0.044^{Dd}	1.003 ± 0.045^{Cd}	0.693 ± 0.051^{Dd}	0.487 ± 0.055^{Dd}	0.564 ± 0.009^{Cd}
5 mg/mL	1.433 ± 0.055^{Cc}	1.140 ± 0.072^{BCc}	0.860 ± 0.044^{Cc}	0.717 ± 0.065^{Cc}	0.682 ± 0.010^{Cc}
2.5 mg/mL	1.803 ± 0.067^{Bb}	1.290 ± 0.050^{Bb}	1.533 ± 0.055^{Bb}	1.487 ± 0.080^{Bb}	1.430 ± 0.079^{Bb}
Paraffin liquid	2.420 ± 0.089^{Aa}	2.620 ± 0.056^{Aa}	2.420 ± 0.062^{Aa}	2.327 ± 0.107^{Aa}	1.860 ± 0.085^{Aa}

Table 8: Effects of thymol on the activity of Ca^{2+}-ATP against *Sarcoptes scabiei in vitro*. The difference between data with the different capital letter within a column is extremely significant (P<0.01), and the difference between data with the different small letters within a column is significant (P<0.05).

Discussion

Sarcoptes scabiei is a global disease that causes substantial losses in many countries [24]. In veterinary clinics, many plant-based acaricidal agents have been exploited and used to control sarcoptic mange. Essential oils and their main constituents are promising alternatives to the use of chemical drugs to control sarcoptic mange [25]. Thymol, a monoterpene found in essential of plants of the families Lamiaceae [26], and research indicates that thymol has an acaricidal activity against engorged females and unengorged larvae of *Rhipicephalus microplus* [27,28], engorged and unengorged females of *Rhipicephalus microplus* [29], and so on. However, this study is the first experiment of the acaricidal activity and acaricidal mechanism of thymol against *Sarcoptes scabiei*.

In this study, thymol killed the mites quickly and effectively and caused 100% mortality in the test within 24 h at 16 mg/mL. However, ivermectin yielded 81.67% mortality after 24 h. The results showed that thymol had a strong acaricidal activity against *Sarcoptes scabiei*. LT_{50} and LC_{50} of the thymol are shown in Tables 2 and 3. Thymol exhibited strong toxicity and could be useful to exploit a new acaricide against the *Sarcoptes scabiei*.

The antioxidant protective enzymes of animals are composed of SOD, CAT, and POD [30], among others. They can eliminate excess oxygen free radicals in the body and play a role in protecting the function and structure of the cell. But drugs or other factor can destroy the equilibrium of the mites and changes in the enzyme system, leading to mites deaths [23]. SOD can remove oxygen-free radicals and form H_2O_2, H_2O_2 remove oxygen-free radicals again and form HO. However, POD decomposes the H_2O_2 into in-noxious substance [23].

Mites treated with the different concentrations of thymol within 24 h, POD and SOD enzyme activity of the mites are gradually inhibited, resulting in the production of excess free radicals and the prevention of the catalytic decomposition of H_2O_2 in the body. Thymol destroys the dynamic equilibrium and induces SOD and POD enzymes activity to kill the mites. Cell energy metabolism was blocked with the decrease of enzymes activity.

MAO is a flavin protease and resides in mitochondrial outer membrane. It can catalyze the deamination of monoamine and plays an important role in regulating several kinds of neurotransmitters with physiological and pathological functions [31]. When MAO is suppressed, the accumulation of monoamine can also cause nerve conduction block. In this paper, the results indicated that the decrease of the MAO activity of the mites treated with thymol caused superfluous sphingosine existing in the body and then nerve conduction was blocked and dead.

GST play a significant role in the cell transportation, protection and exogenous compounds metabolism etc. It catalyzes the synthesis between GSH and the electrophilic groups of the exogenous compounds, and consequently, this process reduces cytotoxicity in the mites [32,33]. It leads to an increased GST activity to improve metabolism and to warrant the normal physiological activities of the organisms when external sources of poison injected into the organism. In the study, thymol increased GST activity in mites compared with control group all the time. Some hydrogen peroxides and endogenous compounds may cause the strong toxicity, leading to the mite death.

The Ca^{2+}-ATP is a transport protein in the cells that remove Ca^{2+} from the cell [34]. Ca^{2+}-ATP enzyme inhibition will cause Ca^{2+} internal flow, then leads to neurotransmitter accumulation. This result showed that Ca^{2+}-ATP enzyme activity was significantly inhibited. Neurotransmitter accumulation caused the blocking of nerves, and finally resulted in mites tissue damage even death.

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Author's Contributions

YCZ performed the majority of the study and analyzed the data, FL and JF contributed to drafting of the manuscript and partial acaricidal study. JHW, QM, RGX, YZ, WS and DY rovided technical assistance. ZHR, ZJZ, ZCZ, GNP, JJD and CT contributed to partial acaricidal study and partial analyses of the data. YCH conceived and designated the study plan, participated in all aspects of the study, provided funds and supervised the research. All authors read and approved the final manuscript.

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