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Research paper

Acaricidal activity and enzyme inhibitory activity of active compounds of essential oils against *Psoroptes cuniculi*

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ABSTRACT

Plant essential oils and its chemical compositions are commonly applied in medicinal and other industries due to their broad advanced pharmacological activities. In the present study, we systematically evaluated the acaricidal activities of twelve compounds of essential oils against *Psoroptes cuniculi in vitro* and *in vivo*. In addition, to support the clinic uses, their toxicities against immortalized human keratinocytes (HaCaT) and human liver cells (HL-7702) and skin irritation were studied for evaluating the liver and skin safety. The possible mechanism of action of certain chemical were investigated by determining the inhibitory activities against cytochrome P450 (P450) acetylcholinesterase (AChE) and glutathione-S-transferase (GST). Among all tested compounds, eugenol exhibited the best acaricidal activity with LC_{50} value of 56.61 μg/ml *in vitro*. Meanwhile, after the treatment of eugenol for five times within 10 days, the *P. cuniculi* were eliminated in the naturally infested rabbits, no skin irritation was found in rabbits treated by eugenol. Moreover, eugenol presented no or weak cytotoxicity against HaCaT cells and HL-7702 cells with IC_{50} values of greater than 100 μg/ml. Furthermore, the moderate inhibitory activities of eugenol against mites P450 and AChE were demonstrated. Above results indicated that eugenol presented the promising acaricidal activity against *P. cuniculi in vitro* and *in vivo,* is safe for both humans and animals at the given doses. This work lays the foundation for the development of eugenol as an environmentally friendly acaricide agent.

1. Introduction

Acariasis is an important ectoparasitic disease caused by mites and infections reduce the yield and quality of afflicted animals (O'Brien, 1999; Dagleish et al., 2007). Particularly in rabbits, *Psoroptes cuniculi* infestation can cause intense pruritus and the formation of crusts and scabs that can completely cover the external ear canal and the internal surfaces of the pinna (Bates, 1999; Nong et al., 2013). Now, rabbit psoroptic mange has become a global disease and resulted in the considerable losses in many countries, such as China, South Korea, United States, Italy, etc. (Fichi et al., 2007; Yeatts, 1994; Eo and Oh-Deog, 2010; Singh et al., 2012).

Now, chemical acaricides have been widely used to treat psoroptic mange, and exhibited relatively satisfactory treatment effectiveness in the veterinary clinic. However, due to acaricide residues, drugs resistance, environmental pollution and other negative public safety effects induced by the overuse of some agents, the use of commercial acaricides has been hindered (Gould, 2010; O'Brien, 1999). The development of alternative and ecofriendly acaricides from sustainable natural products has become increasingly attractive and much needed in agricultural applications (Sun et al., 2017), and many plant-based acaricidal agents have been developed and used to treat and control psoroptic mange in veterinary clinics (Qin and Zhang, 2013; Rosado-Aguilar et al., 2017).

Due to the favorable ecotoxicological properties (e.g., low toxicity to humans, capacity for further degradation, and low environmental impact), plant essential oils have attracted the attention of researchers within the scientific community (Shang and He, 2013; Jia et al., 2018).

Abbreviations: AChE, acetylcholinesterase; ASChI, acetylthiocholine iodine; CDNB, 1-chloro-2,4-dinitrobenzene; CLL, the complementary log-log; DMSO, dimethyl sulfoxide; DTNB, 5,5`-dithiobis (2-nitrobenzoic acid); GST, glutathione-S-transferase; LC_{50}, the median lethal concentration; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PBS, phosphate buffer saline; P450, cytochrome P450; V_{max}, the maximum velocity

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Considering the highly volatile properties of this kind of extracts and their active compounds, they have been used as contact insecticides and fumigants against household pests to protect foods from microorganism contaminations during storage and as herbicides against certain grassy and broad-leaved weeds due to lower residues than other agents (Pisano et al., 2007; Ahuja et al., 2015). The acaricidal activities of essential oils and its chemical compositions against various mites or insects also were reported (Cetin et al., 2010; Monteiro et al., 2017; Ferraz et al., 2010; Gomes et al., 2014; Abdel-Shafy and Zayed, 2002; Xiong et al., 2013). In our previous works, we found that *Rhododendron nivale* essential oil, oregano oil and their major component, carvacrol, thymol and δ-cadinene presented the significant acaricidal activities against *Psoroptes cuniculi* (Shang et al., 2016; Guo et al., 2017). However, the acaricidal activity of compounds of essential oils against *Psoroptes cuniculi* have not been systematically investigated, and their possible mechanism of action are currently unclear

In the present study, we first studied and compared the acaricidal activity of twelve compounds of essential oils against *P. cuniculi in vitro*, and then discuss their structure-activity relationship. Subsequently, the clinical acaricidal efficacy of compound with best acaricidal activity in rabbits was evaluated *in vivo*. For evaluating the safety, the cytotoxicity against two human normal cells *in vitro* and skin irritation potential *in vivo* were studied. Finally, the inhibitory activities of compounds against cytochrome P450 (P450), acetylcholinesterase (AChE) and glutathione-S-transferase (GST) were studied to elucidate the possible mechanism of action.

2. Material and methods

2.1. Chemicals

Chemicals (Fig. 1) were purchased from different companies. δ-Cadinene was purchased from BOC sciences (NY, USA); isoeugenol and 4-allylanisole were purchased from Energy Chemical (Shanghai, China); eugenol acetate, dimethyl sulfoxide (DMSO), α-asarone, carvacrol and thymol were purchased from Meryer Chemical Technology Co. Ltd. (Shanghai, China); 4-allyl-2,6-dimethoxyphenol was purchased from Alfa Aescar (Shanghai, China); eugenol, methyleugenol, acetylthiocholine iodine (ASChI), 5,5`-dithiobis (2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2-H-tetrazolium bromide (MTT), L-reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, USA); chavicol was purchased from Shanghai Zhixin Inc. (Shanghai, China); zingerone and ivermectin were purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China).

2.2. Collection of mites

From the ear cerumen and scabs of naturally infested rabbits, ear cerumen and scabs were collected and then placed in Petri dishes. After incubating the dishes at 35 °C for several minutes, adult *Psoroptes cuniculi* mites with good states were collected under a stereomicroscope (Walton and Currie, 2007). The infested rabbits were treated immediately when the materials were collected. All experiments complied with the rulings of Gansu Experimental Animal Center (Gansu, China) and Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences.

2.3. Acaricidal activity of 12 compounds in vitro

The experiments were carried out according to previously described methods with minor modification (Shang et al., 2013). Compounds were prepared to the different concentrations by 10% DMSO in water. Before the test, three hundred microliters (1000, 500, 250, 100, 50 and 10 μg/ml) of compounds were separately added to culture plates, and filter papers were used to absorb the liquid excess. Ivermectin was used as a positive drug at a range of 50-1 μg/ml, and 10% DMSO in water was added as an untreated control group. Then, 10 adult mites were collected from the naturally infested rabbits ear cerumen and then placed in each well. All plates were incubated at 25 °C under 75% relative humidity. Considering the volatile properties of all compounds, the well was sealed. And at 8 h and 16 h, 50 μl chemicals were added again to each well, respectively. Finally, the mortality was checked after 24 h of treatment. Five replicates were performed for each group.

2.4. Acaricidal activity of eugenol in vivo

According to previously described methods (Guillot and Wright, 1981; Fichi et al., 2007; Nong et al., 2013; Shang et al., 2018), *in vivo* experimental procedures were performed. Firstly, naturally infested rabbits with similar weights, ages and clinical scores were randomly selected and then divided into three groups with five animals in each group, namely, eugenol-treated group, reference group and control group. No significant differences in the clinical scores of rabbits' ears among three groups were found. Considering that mites cannot be concealed without the crusts on the skin surface, the crusts were removed carefully to prevent bleeding and not damage the skin of the ear canal. Before the treatment, the rabbits had not been treated with any acaricides or other drugs, and there are no other complicating diseases in the rabbits.

During the experiment, 1000 μg/ml of eugenol (2 ml) diluted by 10% DMSO in water was sprayed in the external ear canal topically five times within 10 days. Rabbits in the control group were treated with 2 ml of 10% DMSO, and 1% ivermectin was used as positive drug in the reference group. Subsequently, all rabbits' ears were examined to evaluate the presence of scabs, and the scabs were collected to study the presence of mites under a light microscope on 0, 5, 10 and 15 days.

According the following scoring system described by Fichi et al. (2007), the degree of infestation was evaluated: 6 represents all internal surface of the pinna full of scabs with mites; 5 represents 3/4 of the pinna filled with scabs with mites present; 4 was 1/2 pinna filled with scabs and mites; 3 was scabs in ear canal and proximal 1/4 of pinna with mites; 2 was external ear canal filled with scabs and mites; 1 was small number of scabs in the ear canal with mites; 0.5 was small number of scabs but no mites observed; and 0 was absence of scabs and or mites.

2.5. Preliminary safety evaluation

2.5.1. Cytotoxicity test in vitro

According to the described methods (Xu et al., 2016; Shang et al., 2018), the cytotoxicity of eugenol was evaluated. Immortalized human keratinocytes (HaCaT) and human liver cells (HL-7702) were purchased from Procell Life Sci & Tech. Co. Ltd. (Wuhan, China) and were grown in 1640 medium supplemented under a humidified atmosphere of 5% CO_2 in the incubator at 37 °C. Cells were inoculated on 96-well microtiter plates at a density of 5000 cells per well. After treatment with eugenol for 48 h, MTT solution (0.5 mg/ml) was added to each well after the withdrawal of the culture medium and then incubated for 4 h. Then, the result of formazan was dissolved in 150 μL of DMSO after vibration of the culture medium for 10 min. The optical density was measured on a micro plate reader (Multiskan MK3, Thermo Scientific, U.S.A) at 570 nm.

2.5.2. Skin irritant potential

According to the previously described methods (Zhang et al., 2012), skin irritant potential assay. Ten healthy rabbits with similar weights and ages were selected and divided randomly into two groups, control group was treated topically with 10% DMSO, and another group was treated with 2 ml of 1000 μg/ml of eugenol. These agents were applied five times within 10 days according to the method of the acaricidal assay *in vivo*. Subsequently, the skin reaction of all rabbits' ears was

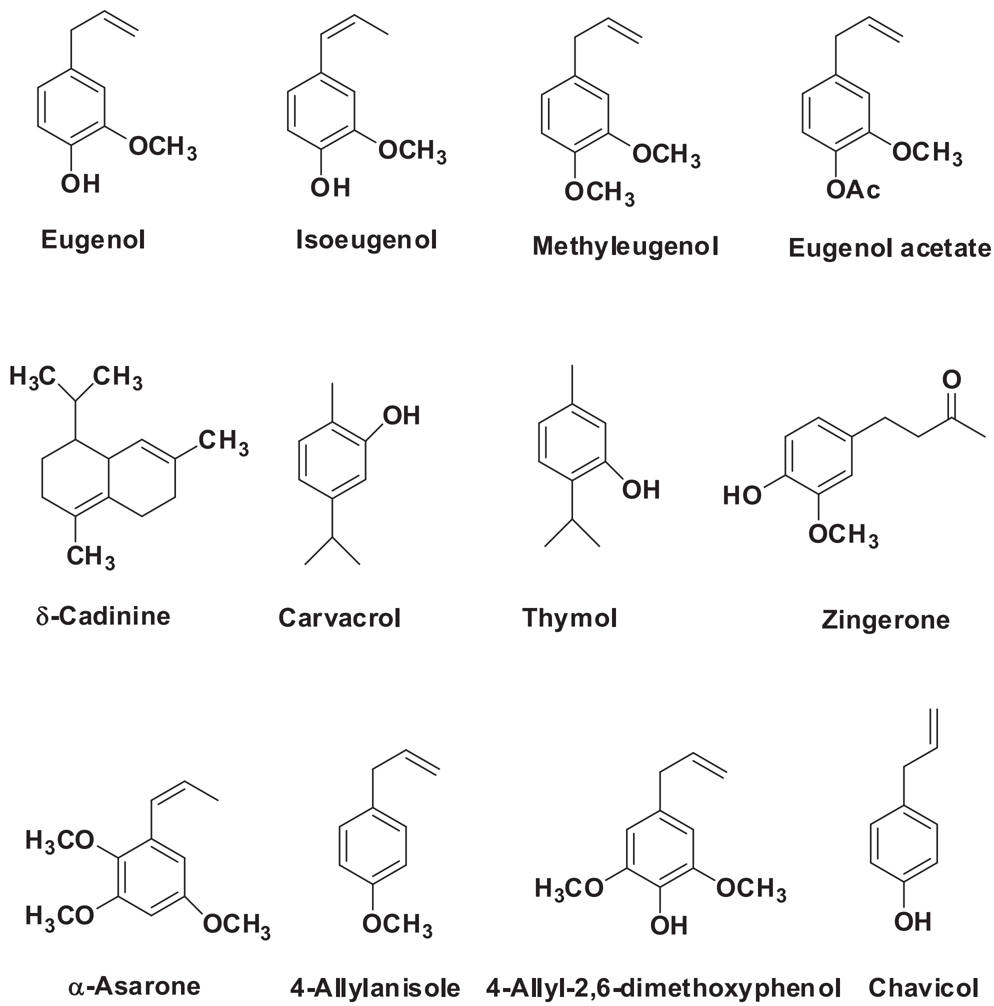

Fig. 1. The chemical structure of 12 compounds of plant essential oils.

examined and recorded on days 0, 5, 10 and 15 days. In additional, we also recorded the skin reactions of infested rabbits' ears in the acaricidal assay *in vivo*.

2.6. Enzyme activities assay

According to the previously described methods (Kang et al., 2013; Kim et al., 2016; Tiwari et al., 2011; Tak et al., 2017), the enzyme inhibitory activities of eugenol were performed. First, 200 mites were collected and placed immediately in a glass homogenizer. After homogenizing for 5 min with 600 μl PBS in ice water, the homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. Finally, the supernatant was collected and used as an enzyme source. The final concentrations of eugenol in each test were used to evaluate its enzyme activities. For evaluating P450 activity, the concentrations were in range of 2–40 μg/ml; for AChE and GST, they were 3.85–76.92 and 3.13–62.50 μg/ml, respectively.

2.6.1. Assay of cytochrome P450 (P450) activity

Thirty microliters of enzyme solution were mixed with 140 μl of 0.05% TMBZ solution, 40 μl of 50 mM potassium phosphate buffer and 10 μl of eugenol in a 96-well plate. Twenty microliters of 3% H_2O_2 was added, and the resulting solution was incubated for 2 h at room temperature. The absorbance was measured at 630 nm, and cytochrome C from equine hearts was used to produce a standard curve. The inhibitory activity was calculated according to the content of P450 s, and the percentage was calculated as (P450 s content of control-P450 s content of treatment)/P450 s content of control - P450 s × 100%.

2.6.2. Assay of acetylcholinesterase (AChE) activity

Firstly, crude protein was incubated with eugenol at 37 °C for 10 min. Then, ASChI (10 μl) and 4 mM DTNB (10 μl) were added to 96-well microplates. The AChE inhibitory activity of eugenol was evaluated by determining the maximum velocity (V_{max}) at 405 nm for 30 min at 40 s intervals.

2.6.3. Assay of glutathione-S-transferase (GST) activity

As well as the method used to assay the activity against AChE, crude protein was incubated with eugenol at 37 °C for 10 min. Then, 100 μl of substrate solution (4 ml of 10 mM reduced glutathione in PBS with 1 ml of 10 mM CDNB) was added to a 96-well microplate. Though determining V_{max} at 340 nm for 30 min at 40 s intervals, the GST inhibitory activity was investigated.

The inhibitory activity (%) was assayed as 100-(V_{max} of treatment/V_{max} of control × 100).

2.7. Statistical analysis

The data obtained were analyzed using one-way ANOVA of SPSS software version 18.0 and expressed as the means ± SD, followed by Student's two-tailed *t*-test to compare the test and control groups. Tukey’s test was used for comparisons of three or more groups. LC_{50} value was calculated using the complementary log-log (CLL) model.

Table 1
The LC_{50} values of 12 compounds of essential oils against *P. cuniculi.*

No.	Compounds	LC_{50}* (μg/ml)	Regression line	95% CI** (μg/ml)	Pearson Chi-square
1	Eugenol	56.61	Y = 2.458X-4.309	23.20–109.09	35.197
2	Isoeugenol	108.05	Y = 1.832X-3.726	61.12–177.88	18.476
3	Methyleugenol	158.28	Y = 1.681X-3.696	33.87–770.62	66.445
4	Eugenol acetate	63.08	Y = 1.594X-2.869	2.71–270.09	74.966
5	δ-Cadinine	> 500	–	–	–
6	Carvacrol	336.51	Y = 1.474X-3.726	159.33–1172.02	29.657
7	Thymol	197.84	Y = 1.265X-2.904	–	92.110
8	Zingerone	> 500	–	–	–
9	α-Asarone	112.98	Y = 4.002X-8.217	–	–
10	4-Allylanisole	> 500	–	–	–
11	4-Allyl-2,6-dimethoxyphenol	> 500	–	–	–
12	Chavicol	> 500	–	–	–
13	Ivermectine**	3.53	Y = 2.246X-1.230	2.61-4.65	8.153

* LC_{50} was analyzed according to the mortality (%) of mites at 24 h.
** CI, Confidential interval.

3. Results

3.1. Acaricidal activity of chemicals in vitro

Among twelve compounds, eugenol presented the strongest acaricidal activities against *P. cuniculi in vitro,* with a median lethal concentration (LC_{50}) value of 56.61 μg/ml at 24 h. The mean mortalities were 100%, 100%, 100%, 71.67%, 26.67% and 10% at the concentration of 1000 μg/ml, 500 μg/ml, 250 μg/ml, 100 μg/ml, 50 μg/ml and 10 μg/ml, respectively. The second strongest anti-*P. cuniculi* compound was eugenol acetate, with a LC_{50} value of 63.08 μg/ml. δ-Cadinene, zingerone, 4-allylanisole, 4-allyl-2,6- dimethoxyphenol and chavicol exhibited weak acaricidal activities, with LC_{50} values > 500 μg/ml at 24 h. The LC_{50} value for the positive control, ivermectin, was 3.53 μg/ml against mites (Table 1) Repeated administration within 24 h would decrease the LC_{50} values of chemicals against mites.

3.2. Acaricidal activity of eugenol in vivo

Subsequently, because it exhibited the highest acaricidal activity against *P. cuniculi in vitro*, the acaricidal activity of eugenol was evaluated *in vivo*. After eugenol topical treatments, the number of scabs on the ears were substantially decreased. From Table 2, we can see that the clinical scores of infestations at days 5 and 10 were 3.1 and 0.5, respectively, and only small scabs existed in the ear canals at days 10 and 15. In addition, eugenol treated rabbits were free of mites, and showed a positive mental and physical status with normal movement ability, good appetite and good fur and skin condition at end of the test ($P < 0.01$) (Table 2). These results suggested that eugenol had significant acaricidal activity *in vivo* and might be used in clinic settings to control psoroptic mange.

Table 2
The acaricidal activity of eugenol against *Psoroptes cuniculi* in rabbits *in vivo*, measured by clinical score of infestation.

Groups	Days ± SD			
	0-day	5-day	10-day	15-day
Eugenol	3.9 ± 0.45A	3.1 ± 0.19A	0.5 ± 0.15A	0.2 ± 0.12
Ivermectin	3.9 ± 0.45A	1.1 ± 0.25B	0.00 ± 0.00B	0.0 ± 0.00
Control	4.1 ± 0.38A	4.0 ± 0.40C	4.2 ± 0.00C	–*

The difference between data with the different capital letter within a column is significant ($P < 0.01$).
* After the observation at day 15, rabbits of negative control were treated.

3.3. Preliminary safety evaluation

3.3.1. Cytotoxicity

In the current studies, immortalized human keratinocytes (HaCaT) and liver cell lines (HL-7702) were employed to evaluate the skin and liver cytotoxicity of eugenol *in vitro*, respectively. We demonstrated that eugenol was inactive and had weak cytotoxicity against HaCaT cells and HL-7702 cells, respectively, with IC_{50} values greater than 100 μg/ml (Fig. 2).

3.3.2. Skin irritation

During the experimental periods, no skin irritation was found in healthy and naturally infested rabbits treated by eugenol, such as allergy, inflammatory response and irritation. The clinical scores of skin irritant potential in all groups were zero (Table 3). The above results indicate that at the given concentrations, eugenol was safe and might have potential for clinical uses. However, further safety evaluations should also be conducted.

3.4. Enzyme inhibitory effects

3.4.1. Effect on P450s activity

In this paper, the inhibitory activities of eugenol against P450, GST and AChE were assayed to investigate the modes of action. As presented in Figure 4 A, after treatment with eugenol, the P450 s activity of mites was inhibited compared to the control with dose-dependent manner; the percentage decreases were 9.28%, 3.41%, 15.47%, 28.37%, 33.67%, 38.07% and 42.79% at the concentrations of 2.00 μg/mL, 4.00 μg/mL, 8.00 μg/mL, 16.00 μg/mL, 24.00 μg/mL, 32.00 μg/mL and

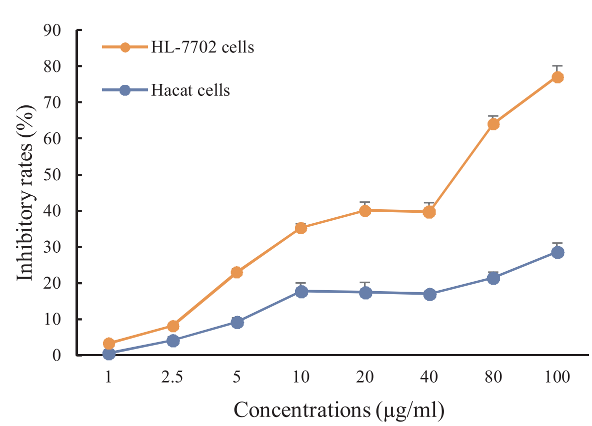

Fig. 2. The cytotoxicity of eugenol against immortalized human keratinocytes (HaCaT) and liver cell lines (HL-7702).

Table 3
The skin irritant potential of eugenol.

Group	Skin irritant potential (15 days)	
	Healthy rabbits	Naturally infested rabbit
Eugenol group	No irritation	No irritation
Control group*	No irritation	No irritation

* Control group was treated with 10% DMSO.

40.00 μg/mL, respectively.

3.4.2. Inhibition of AChE activity

As presented in Figure 4C, eugenol exhibited moderate mites AChE inhibitory activity without the dose-dependent manner. At the concentrations of 3.85 μg/mL, 7.69 μg/mL, 15.38 μg/mL, 30.77 μg/mL, 38.46 μg/mL and 76.92 μg/mL, the inhibition rates were 22.68%, 30.80%, 40.68%, 39.08%, 34.55% and 34.35%, respectively.

3.4.3. Inhibition of GST activity

From Figure 4B, it can be observed that eugenol exhibited the weak *P. cuniculi* GST inhibitory activities with inhibition rates of 0.76%, 3.63%, 4.32%, 9.46%, 13.80% and 16.37% at the concentrations of 3.13 μg/mL, 6.25 μg/mL, 12.50 μg/mL, 25.00 μg/mL, 31.25 μg/mL and 62.50 μg/mL, respectively.

4. Discussion

Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound, which is a major component of essential oils of several plants in a family of Myrtaceae (Prakash and Gupta, 2005). As a natural aromatic substance, eugenol presents a remarkable broad spectrum of activities (Nagababu et al., 2010), including antiparasitic, antioxidant, anesthetic, antibacterial, antifungal, antiviral and other activities (de Morais et al., 2014; Mastelić et al., 2008; Shang and He, 2013; Al Wafai et al., 2017; Benencia and Courreges, 2000). Hence, eugenol is widely applied in multiple products of various industries, such as food flavoring and fragrance (Shang and He, 2013). In our current work, we found that eugenol has the best acaricidal activity among all of the tested compounds *in vitro* (Table 1). Moreover, it cured the rabbit`s mange induced by *Psoroptes cuniculi in vivo*. The further structure-activity relationship analysis indicated that the presence of hydroxyl groups played an important role in the acaricidal activity of compounds; and the o-methoxy group and p-allyl group also would influence the toxicity against mites (Fig. 2).

Although eugenol has been used extensively in fragrances and flavor formulations since 1900 (Rothenstein et al., 1983), it is important to re-evaluate its safety as a potential acaricide agent for controlling psoroptic manage by spraying it on skin surface (transdermal administration), especially regarding its skin irritant and cytotoxicity. Additional studies showed that eugenol, at the given concentrations, is safe for the animal use, which suggests that it could be used in clinical settings (Fig. 2). The low cytotoxic activity also was reported by Prashar et al. (2006). To advance the application of eugenol in veterinary clinics, we investigated the mechanisms of action by determining the inhibitory effects against three enzymes.

Elucidation of the mode of action of naturally derived acaricides is of practical importance for mite control because it may provide useful information on the most appropriate formulation and delivery means. As the most important enzymes among phase I and phase II reactions, P450 s participate in oxidation (Bass et al., 2011), and GST participate in the detoxification of drugs and other agents in insects and mites (Mounsey et al., 2010; Yu and Abo-Elghar, 2000). From Fig. 3, we can see that eugenol significantly inhibited the P450 s activity in a dose-dependent manner up to 42.79%. However, it was weakly inhibitory to

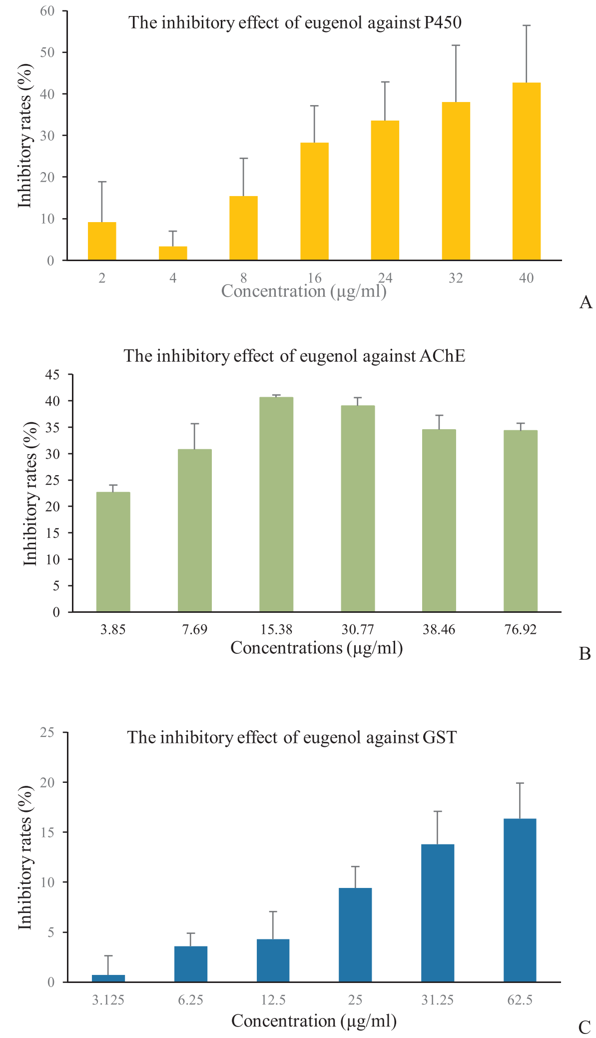

Fig. 3. P450, AChE and GST activities of mites after treatment with eugenol at different concentrations (A. The inhibitory effect against CYP450 activity; B: The inhibitory effects against AChE activity; C: The inhibitory effects against GST activity).

GST activity. Considering that pesticides generally possess several insecticidal mechanisms including disruption of normal metabolism by interfering with neuron transmission, the inhibitory activity against AChE was assayed which is related to the neural conduction and movements of mites (Shang et al., 2017). In our studies, the moderate inhibitory effect against AChE activities was showed (Fig. 3). These results indicated that eugenol inhibited P450 s activity in the phase I reaction to further induce insect death. During the treatment of eugenol, neural conduction of mites may be blocked by inhibiting AChE activity.

5. Conclusion

In conclusion, eugenol exhibited significant acaricidal activity against *P. cuniculi in vitro*. The good clinical efficacy *in vivo* and safety also were demonstrated. Future studies will focus on finding relatively safer acaricides by designing and synthesizing a series of eugenol derivatives and understanding the mechanisms of action of these compounds. In addition, certain chemical drawbacks of eugenol should also be improved, such as its volatile properties. More tests should be carried out at different times or places for solving the questions induced by high volatile properties and producing more convincing data. This study lays an important foundation for the future development of eugenol as a relatively safe acaricide in agricultural applications.

Competing interests

All authors declare that they have no competing interests.

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