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Research paper

The acaricidal activity and mechanism of eugenol on *Psoroptes cuniculi*

Wuren Ma[1], Yunpeng Fan[1], Zengyuan Liu, Yuanjie Hao, Yuan Mou, Yingqiu Liu, Weimin Zhang, Xiaoping Song*

College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, PR China

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ABSTRACT

In this study, the acaricidal effect of eugenol was measured and its mechanism of action investigated. The results showed that eugenol possessed the effect of killing *Psoroptes cuniculi*, and could regulate the mRNA expression of glutathione S-transferase (GST), catechinic acid (Ca) and thioredoxin (Trx). PPAR, NF-kappa B, TNF, Rap 1 and Ras signaling pathways might be the main pathways that involved into the process of killing mites. These findings suggested that eugenol could be developed into a new kind of acaricide, and further expand current knowledge on the mechanisms of eugenol for killing *Psoroptes cuniculi* of eugenol.

1. Introduction

Psoroptes cuniculi parasitizes the external auditory canal of rabbits and feeds on serous exudate, secretions and blood (Bates, 1999). This disease has a high transmission rate and spreads rapidly, which seriously endangers the healthy development of rabbit cultivation industry (Zhang et al., 2013). *Psoroptic acariasis* is caused by Psoroptidae, mainly including *chorioptes* and *otodectes*. Many varieties of animals can be infected, such as pig, horse, cow, sheep and rabbit (Song et al., 2002). *Psoroptes* development includes 4 stages, ovum, larva, nymph and adult mites. Male and female mites have two nymphal stages. The eggs of the mite develop about 12 days, the spawning period of the female mite is about 40 days, and can be completed within 55–60 days (Chen et al., 2012; Sanders et al., 2000; Vercruysse et al., 2006).

At present, the treatment of acariasis in animals is still dominated by synthetic drugs such as macrocyclic lactones, organophosphorus and pyrethroids, but the use of chemicals could result in resistance in target species, toxicity and environmental hazards (Borges et al., 2013). Therefore, natural plant drugs with high efficiency, low toxicity and easy degradation have become of interest (Fichi et al., 2007; Hu et al., 2015).

In previous research, our laboratory found that *Eugenia caryophyllata* had significant acaricidal activity, and the highly effective acaricidal active ingredient eugenol was isolated and purified (Ruan, 2005). In the current experiment, we studied the acaricidal effect of eugenol on *Psoroptes cuniculi*, and investigated its mechanism of action, the aim is to provide theoretical guidance for clinical application of eugenol.

2. Materials and methods

2.1. Collection of Psoroptes cuniculi

Rabbits were purchased from Yabai rabbit warren and were natural infected with *Psoroptes cuniculi*, the average weight was 2.8 ± 0.8 kg. Mites were collected from the scab of rabbit's auricle. The activity of mites was evaluated when placed on water bath at 35 °C for 30 min. After morphological identification, mites with normal vitality were selected for testing.

2.2. The effect of eugenol in killing Psoroptes cuniculi

Four hundred *Psoroptes cuniculi* were randomly allocated into one of four groups. They were treated with eugenol (purity ≥99%, No. 140943, which was purchased from Alading Co., Ltd.) at different concentrations (4, 2 and 1 mgmL^{-1}), and in blank control (BC) group they were treated with liquid paraffin (No. 20130905, Bodi Chemical Co., Ltd.). Each group was allocated to one of five replicates with 20 mites in each. At 1, 2, 3, 4, 6, 8, 12, 18 and 24 h after treatment, the mites were observed under the microscope, the number of deaths recorded and mortality calculated (Mites were considered dead when their limbs were immobile and non-responsive.).

Abbreviations: BC, blank control; GST, glutathione S-transferase; Ca, catechinic acid; Trx, thioredoxin; KOG, eukaryotic orthologous group; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

* Corresponding author at: College of Veterinary Medicine, Northwest A&F University, Yangling, 712100, PR China.
E-mail address: sxpxbnl@163.com (X. Song).

[1] Both authors contributed equally to this work.

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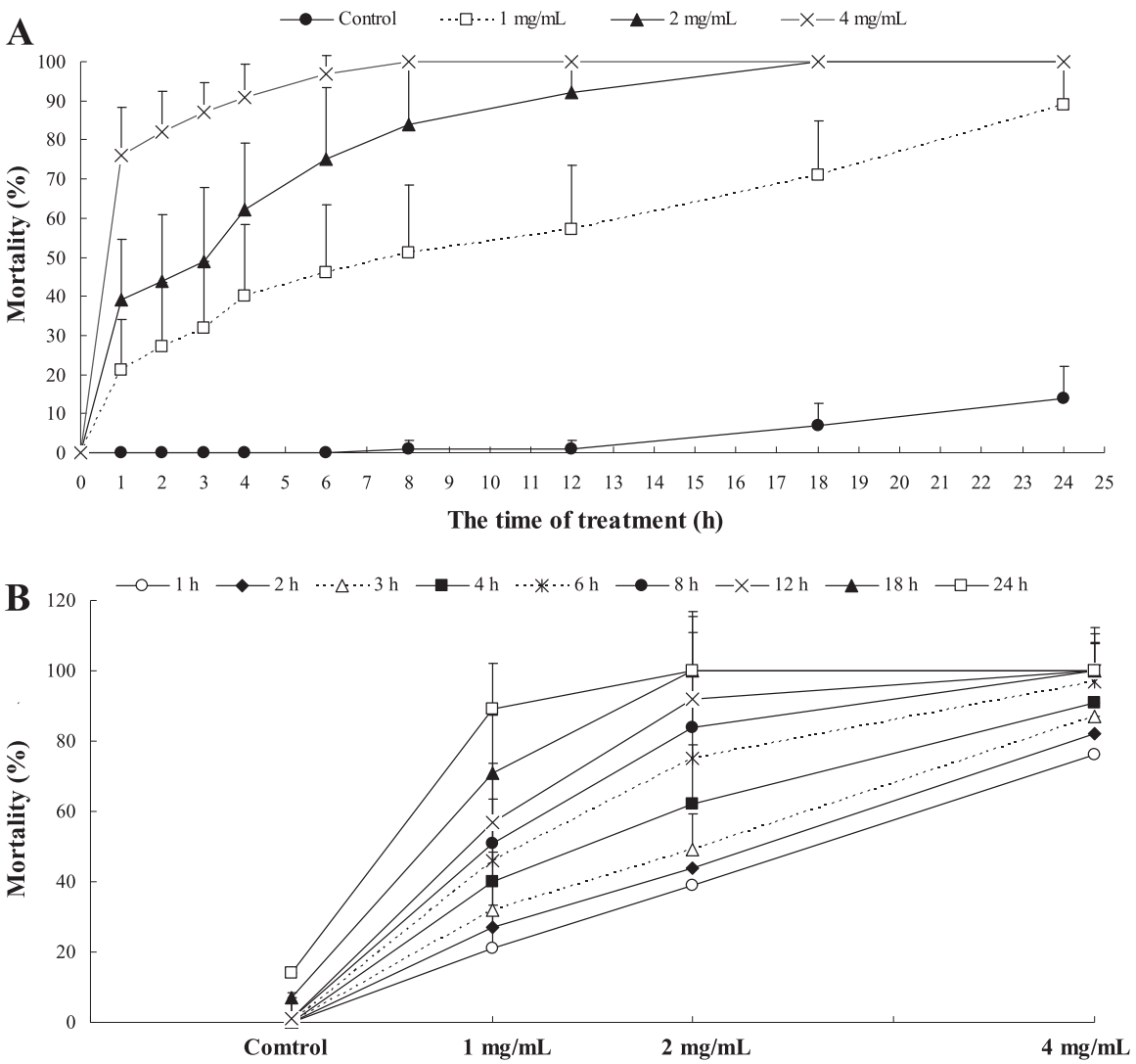


Fig. 1. The effect of eugenol on killing *Psoroptes cuniculi.*
A, the effect of eugenol at different time points on killing *Psoroptes cuniculi*; B, the effect of eugenol at different concentrations on killing *Psoroptes cuniculi*.

2.3. The effect of eugenol on the mRNA expression of glutathione S-transferase (GST), catechinic acid (Ca) and thioredoxin (Trx) gene in Psoroptes cuniculi

Additional four hundred *Psoroptes cuniculi* were randomly allocated to one of four groups. They were treated with eugenol at 4, 2 and 1 $mgmL^{-1}$, and in BC group they were treated with liquid paraffin. At 0.5, 1, 2, and 3 h after treatment, total RNA of mites was isolated according to manufacture's protocol of Trizol kit (TakaRa) and reverse transcription was performed with a final volume of 10 μL.

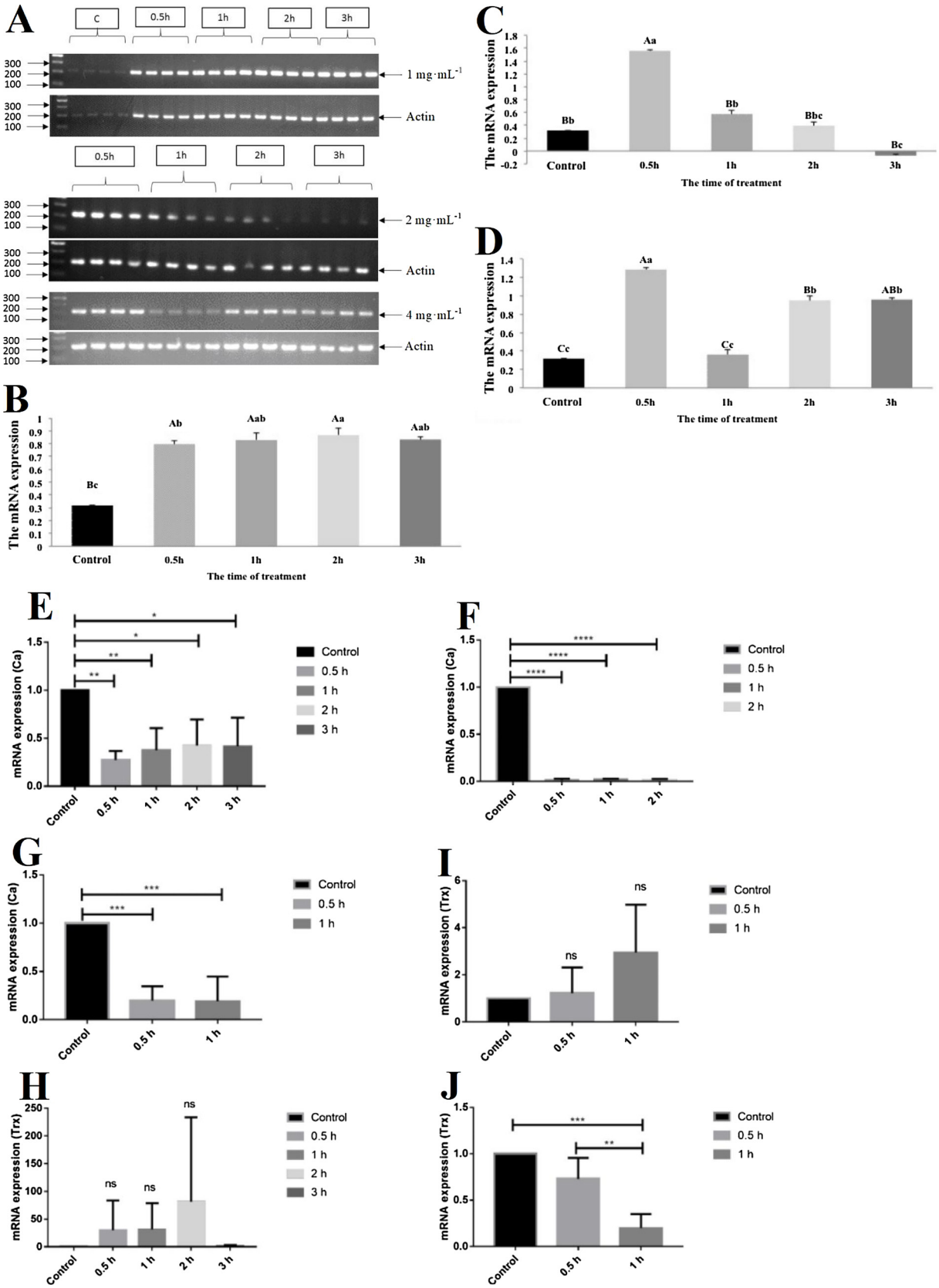
The primers sequences of GST, Ca, Trx and actin were synthesized by Shanghai Invitrogen Bio Technologies Co. Ltd. The primer sequences were listed as follow: GST, forward: 5′-TGGGATATCCGTGGTTTAGG-3′, reverse: 5′-TGTGACCCAAG TATCGGAGA-3′; Ca, forward: 5′-GTACGA CAAGCACAA GCACC-3′, reverse: 5′-CCTCAGCTTCTAAACGGCGA-3'; Trx, forward: 5′-GTT GTGGCCGTATCAG TGGA-3′, reverse: 5′-CTGAT CGGCCGACTGGTAAA-3′; actin, forward: 5′-AAGTCATCTCCATCGGT TCG-3′, reverse: 5′-GATACGATCGGCAATACCT -3′. Amplification was carried out in a total volume of 20 μL containing 1 μL of transcribed cDNA, 1 μL of each specific primer, 7 μL of ddH2O, and 10 μL of All-in-One PreMix. PCR was performed for 30 cycles using a PTC-200 thermal cycler (Bio-Rad Laboratories, Inc) with the program of initial denaturation at 94 ℃ for 3 min, denaturation for 30 s, annealing for 30 s, and elongation at 72 ℃ for 10 min. Semi-quantitative RT-PCR was performed using actin as an internal control to normalize gene expression. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing goldview, and the amplified bands were visualized and photographed using JS-680B Gel Documentation and Analysis System (Shanghai Peiqing Science and Technology Co., Ltd.).

2.4. Analysis of transcriptome of eugenol on killing Psoroptes cuniculi

Psoroptes cuniculi were treated with eugenol at 2 $mgmL^{-1}$ for 2 h, and then the differential expression of the transcriptome of mite was analyzed. Both the BC and eugenol groups were tested in parallel for 3 groups. After the treatment of the samples, the transcriptome was analyzed by Gene Denovo Co., Ltd.

2.5. Statistical analysis

The mortality of mite was calculated using the formula. The mortality (%) = the number of death mites/ the total mites ×100%. After statistical analysis, 5 repeated data at all time points and concentrations were expressed as the mean ± S.D. Duncan's multiple range test was used to determine the differences among groups with the software SPSS 20.0. The median lethal dose (LD50) of eugenol at each time point was



(caption on next page)

Fig. 2. The mRNA expression of GST, Ca and Trx in *Psoroptes cuniculi*.
A, Electropherotypes of different concentrations of eugenol treated for psoroptes cuniculi at different times; B, The gray analysis results of GST treated with eugenol at 1 mg mL^{-1}; C, The gray analysis results of GST treated with eugenol at 2 mg mL^{-1}; D, The gray analysis results of GST treated with eugenol at 4 mg mL^{-1}. E, The gray analysis results of Ca treated with eugenol at 1 mg mL^{-1}; F, The gray analysis results of Ca treated with eugenol at 2 mg mL^{-1}; G, The gray analysis results of Ca treated with eugenol at 4 mg mL^{-1}. H, The gray analysis results of Trx treated with eugenol at 1 mg mL^{-1}; I, The gray analysis results of Trx treated with eugenol at 2 mg mL^{-1}; J, The gray analysis results of Trx treated with eugenol at 4 mg mL^{-1}. The values are presented as means ± SD (n = 4). Different upper case letters superscripts indicate extremely significant differences ($p < 0.01$), and different lower case letters superscripts indicate significant differences ($p < 0.05$). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0002$, ****: $p < 0.0001$.

calculated by improved Karber's method.

3. Results

3.1. The effect of eugenol on killing Psoroptes cuniculi

The effect of eugenol at different time points on killing *Psoroptes cuniculi* is illustrated in Fig. 1A. The result showed that some mites could be killed after treated 1 h at three concentrations. The mortality in 4 $mgmL^{-1}$group was the highest, accounting for 76%. The mites were all killed in 4 $mgmL^{-1}$ after being treated for 8 h. The mites were all killed in 2 $mgmL^{-1}$ at 18 h. In addition, the LD50 of eugenol at 1, 2, 3, 4, 6, 8, 12, 18 and 24 h after treatment were 1.564 ± 0.023, 1.575 ± 0.023, 1.575 ± 0.023, 1.459 ± 0.022, 1.390 ± 0.021, 1.324 ± 0.019, 1.227 ± 0.017, 1.106 ± 0.014 and 1.039 ± 0.009 respectively. These results indicated that the mortality of mites increased, and the LD50 decreased with the prolonging of time.

The effect of eugenol at different concentrations on killing *Psoroptes cuniculi* is shown in Fig. 1B. The result showed that there was no significant difference from 1 h to 12 h in the control, which indicated that the activity of mites was normal within 12 h. The mortality of mites increased with time at three concentrations from 1 h to 12 h. There was no significant difference in 2 $mgmL^{-1}$ group from 8 h to 24 h, and in 4 $mgmL^{-1}$ group from 4 h to 24 h. The result indicated that the effect of eugenol at 4 $mgmL^{-1}$ on killing mites was the fastest ($p = 0.003$).

3.2. The effect of eugenol on the mRNA expression of GST in Psoroptes cuniculi

As shown in Fig. 2A–D, the mRNA expressions of GST in 0.5, 1, 2 and 3 h groups were greater than control group after treated with eugenol at 1 $mgmL^{-1}$ ($p = 0.000$). The mRNA expressions in 0.5 h group were the highest, and greater than othtr groups after treated with 2 and 4 $mgmL^{-1}$ ($p = 0.002$). The mRNA expressions in 2 and 3 h groups were greater than 1 h and control groups ($p = 0.001$).

3.3. The effect of eugenol on the mRNA expression of Ca

As shown in Fig. 2E–G, the mRNA expressions of Ca in 0.5, 1, 2 and 3 h groups were less than control group after treated with eugenol at 1 $mgmL^{-1}$ ($p = 0.010$). The mRNA expression in 0.5, 1 and 2 h groups were less than control group after treated with 2 $mgmL^{-1}$ ($p = 0.000$). The mRNA expression in 0.5 and 1 h groups was less than control group after treated with 4 $mgmL^{-1}$ ($p = 0.000$).

3.4. The effect of eugenol on the mRNA expression of Trx

As shown in Fig. 2H–J, the mRNA expression of Trx in control and 0.5 h groups were greater than 1 h group after treated with 4 $mgmL^{-1}$ ($p = 0.000$ and 0.002, respectively). There was no significant difference in 0.5, 1 and 2 h groups at 1 and 2 $mgmL^{-1}$.

3.5. The analysis result of transcriptome of eugenol on killing Psoroptes cuniculi

3.5.1. The expression analysis of differential genes

The number of expressed genes was 26,686 in the control group, and in eugenol group was 27,905. In the further analysis of the differential genes, 572 up-regulated genes and 157 down-regulated genes were observed, which is shown in Fig. 3A, B. In addition, the expression level in eugenol group was significantly up-regulated compared with the control group.

3.5.2. KOG analysis

As shown in Fig. 3C, a total of 28,118 unigenes were annotated into 26 groups of KOG. T group (Signal transduction mechanisms) had the greatest distribution of unigenes, which reached 5367. The second was R group (General function prediction only), which reached 4162. The third was O group (Posttranslational modification, protein turnover, chaperones), which reached 2612.

3.5.3. GO functional analysis

8612 unigenes were annotated to 48 functional categories, there were 3873 genes in biological processes, 2171 in molecular functions and 2568 in cellular components (Fig. 3D). 25 down-regulation pathways were related to cell components, molecular functions and biological processes. Among them, 12 pathways were related to cell components, 7 were related to molecular functions, and 6 were related to biological processes. At the same time, 15 pathways with up-regulated genes were obtained, 6 pathways were related to cell components, 3 were related to molecular functions, and 6 were related to biological processes (Fig. 3E).

3.5.4. KEGG functional analysis

A total of 20 up-regulated pathways and a total of 20 down-regulated pathways were observed. The main up-regulated pathway was PPAR signaling pathway. The main down-regulated pathways were associated with NF-kappa B, TNF, Rap 1 and Ras signaling.

4. Discussion

In this study, eugenol at different concentrations was used to kill *Psoroptes cuniculi*. The results indicated that the acaricidal effect of eugenol had obvious time and dose dependent, and had the characteristics of high activity and rapid efficacy, which possessed important guiding significance for its clinical application.

GST is an important detoxification protein in *Psoroptes cuniculi*. Ca is related to the movement of mites, and Trx is a highly conserved protein with low molecular weight, which is involved in redox reaction (Ahn et al., 2011; Niu et al., 2012). In this experiment, the acaricidal activity and mechanism of eugenol were studied from the aspects of detoxification, movement and oxidative stress systems of mites. Therefore, three genes of GST, Ca and Trx were selected and analyzed.

It has been proved that the enzyme activity of GST was significantly inhibited after the treatment of mites with 0.6% eugenol (Xiong et al., 2013). GST belongs to phaseII superfamily enzyme, which codes for multiple genes, is widely distributed, and participates in metabolic reactions. It plays important roles in detoxification, antioxidant and

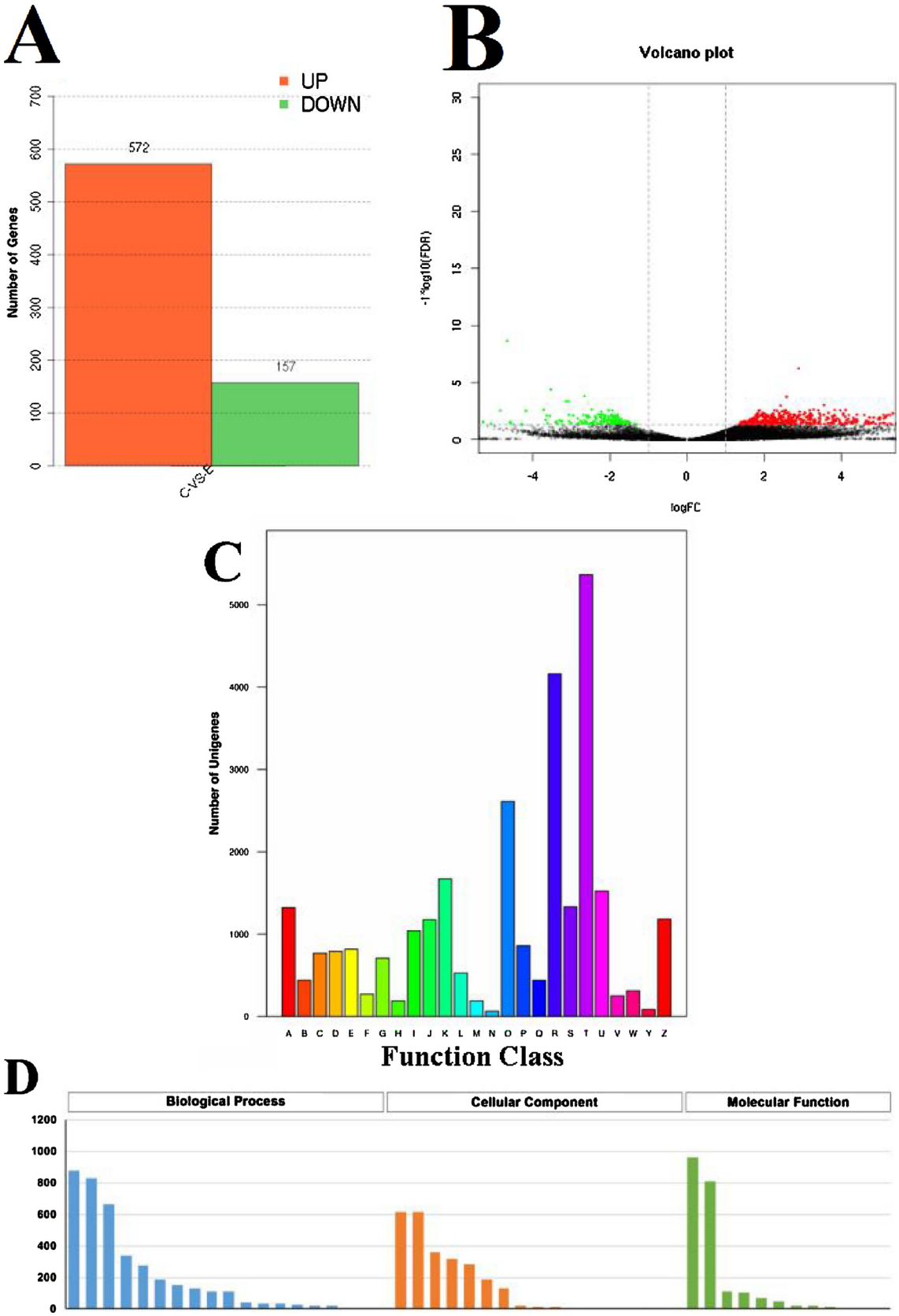


Fig. 3. The analysis results of transcriptome of eugenol on killing *Psoroptes cuniculi*.
A, The expression analysis of up-regulated and down-regulated genes after treatment with eugenol; B, The volcano diagram of differential genes after treatment with eugenol; C, The KOG classification of total unigenes after treatment with eugenol; D, The GO categories of total unigenes after treatment with eugenol; E, The GO functional analysis of up-regulated and down-regulated genes after treatment with eugenol.

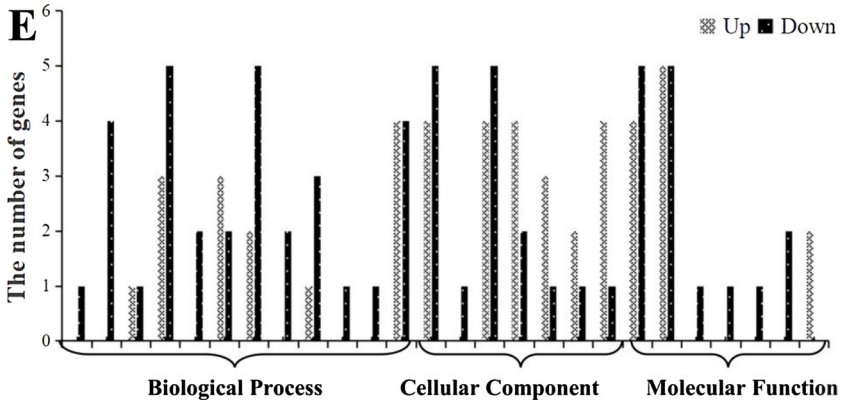


Fig. 3. (*continued*)

metabolic regulation (Niu et al., 2012). As a highly efficient system of detoxification metabolism, GST can catalyze the conjugation of reduced glutathione with the electrophilic groups of exogenous toxic substances to discharge the toxins out of the body, thereby reducing cytotoxicity. In addition, the inhibition of GST can lead to the death of mites because of the loss of detoxification function of mites. In this experiment, the mRNA expression of GST initially increased, then decreased and then rose after treating mites with eugenol. In general, the activity of GST in drug groups showed a tendency to slowly decrease. It indicated that eugenol at a certain concentration could down-regulate the mRNA expression of GST in *Psoroptes cuniculi*. The pattern of activity observed here showed a similar profile to that reported by Xiong et al (2013) following treatment of rabbit itch mite with 0.6% eugenol who also reported lesser activity than a liquid paraffin treated control group at all time points, indicating eugenol inhibits the enzyme activity of GST.

The mRNA expression of Ca and Trx were different from controls after treatment with different concentrations of eugenol. Ca possesses antioxidant properties, it could scavenge reactive oxygen. Trx is a ubiquitous protein regulator of redox reaction in all living cells. It operates together with Trx reductase and NADPH as a general protein disulfide catalytic system, playing an important role in several biological processes (Ahn et al., 2011; Drechsel and Patel, 2010). The results indicated that ugenol could kill mites through the motor system and oxidative stress system.

In order to further study the mechanism of eugenol on acaricidal effect, transcriptome sequencing was carried out, with the aim to explore the important regulatory gene which was related to the acaricidal activity of eugenol from the transcriptome level. Transcriptome analysis is able to study the differential expression of transcriptional product classification, genome functional elements, transcriptional structure and post-transcriptional modification (Ozsolak and Milos, 2011). At present, High-throughput sequencing technology has been widely used in the transcriptome studies of animals, plants and microorganisms (Nagalakshmi et al., 2008; Wang et al., 2017). The results showed that 25 pathways associated with down-regulation genes and 15 pathways associated with up-regulation genes were obtained, which was related to cell components, molecular functions and biological processes. In addition, PPAR, NF-kappa B, TNF, Rap 1 and Ras signaling pathways may be the main pathways. The results indicated that these signaling pathways played an important role in killing *Psoroptes cuniculi* of eugenol. But, the reason that eugenol induced the significant changes of these signaling pathways in *Psoroptes cuniculi* remains to be further investigated.

5. Conclusion

Eugenol had the effect on killing *Psoroptes cuniculi*, possibly through regulating the mRNA expression of GST, Ca and Trx, and PPAR, NF-kappa B, TNF, Rap 1 and Ras signaling pathways. These findings indicated that eugenol could be developed into a new kind of acaricide, and further expanded current knowledge on the mechanisms of killing *Psoroptes cuniculi* of eugenol.

Conflict of interest

There is no any conflict of interest.

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