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Toxicity of Stigmasterol Isolated from Crofton Weed, *Eupatorium adenophorum* Spreng. Against a Rabbit Ear Mite, *Psoroptes cuniculi*

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Authors' Contribution
XN performed the majority of the experiments, analyzed the data and drafted the manuscript. GYY provided technical assistance. YJY, FZC, TM and GW contributed to investigations of acaricidal activity.

Key words
Silica column chromatography, ^{1}H-NMR, ^{13}C-NMR, Stigmastero.

ABSTRACT

In order to develop a new type of insecticide, this study lays foundation for the development of stigmasterol from the *Eupatorium adenophorum* Sprengas nontoxic and pollution-free pesticides that can be released to the environment. The toxicity test of stigmasterol against *Psoroptes cuniculi in vitro* showed that stigmasterol was toxic under 0.5% concentration. Treatment under this condition also showed mite mortality of 16.67% within 12 h and 23.33% within 24 h. The insecticidal activity of stigmasterol was weaker than that of ageraphorone compound obtained from *E. adenophorum* Spreng. Through preliminary separation. However, in the experiments, we found an interesting phenomenon, that is, the dead state of mites treated with stigmasterol was similar to that of mites of the positive control group treated with fenvalerate. Furthermore, the dead state of mites treated with stigmasterol was different from that of mites of the negative control group. This result confirmed the insecticidal activity of stigmasterol. We conducted a thorough research on the insecticidal molecular mechanism of stigmasterol to lay the foundation for the development of stigmasterol as nontoxic and pollution-free pesticides that can be introduced into the environment.

INTRODUCTION

Eupatorium adenophorum Spreng. is the most harmful invasive species in China, especially in the southwest of China. This species causes local ecological destruction, and thus China has been attempting to control this harmful weed. However, it has not been effectively controlled because of its fast spreading characteristic. In recent years, *E. adenophorum* Spreng. has been developed as a potential botanical pesticide, and efforts have been made toward profitable utilization of its waste. Sesquiterpenoids from this weed have hepatotoxicity and possess insect repellent and insecticidal activity (Bhardwaj *et al*., 2001; Zou *et al*., 2009; Li *et al*., 2015). Moreover, its sesquiterpenoid components showed toxicity to lice and scabies, and its dotriacontane alcohol has mite removal effect. The results of the present study will provide a theoretical basis for reasonable development and utilization of *E. adenophorum* Spreng.

The finding of this study combined with that of an early research showed that petroleum ether extracts contain biological activity against *P. cuniculi* and *Chorioptes* spp. (Acari: Psoroptidae) (Nong *et al*., 2012, 2014; Liu, 2013). Silica column chromatography was conducted to separate a composition from petroleum ether extract of *E. adenophorum* Spreng. Hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) were used to determine the stigmasterol content of this composition. The toxicity test of stigmasterol against *P. cuniculi* in vitro was conducted. This study lays foundation for the development of stigmasterol as nontoxic and pollution-free pesticides that can be released to the environment.

MATERIALS AND METHODS

E. adenophorum Spreng. samples were collected from Xichang City in Sichuan Province. The samples were naturally dried, and were then crushed and stored for later use. The adult *P. cuniculi* individuals were isolated from the ear cerumen of naturally infected rabbits. The samples

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were collected and placed in Petri dishes, and were then incubated at 35°C for 30 min in an incubator. Afterward, the adult mites were collected and used for testing (Walton and Currie, 2007).

Stigmasterol separation was conducted according to the method of Nong *et al.* (2012, 2014). The compound structure was elucidated by ^{1}H-NMR and ^{13}C-NMR data (Wang *et al.*, 2007). ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Varian Unity INOVA 400/45 NMR spectrometer in $CDCl_3$ with TMS as the internal standard.

Experimental procedures were performed as previously described by Nong *et al.* (2014). The stigmasterol compound was diluted with 1:1 of distilled water and glycerol to obtain three different experimental concentrations (0.5, 0.25, and 0.125 m/v). Each concentration of the stigmasterol extract (1 mL) was added into six-well cell culture plates (Costar 3516) containing two filter paper chips to absorb the liquid. A total of 10 adult *P. cuniculi* individuals were placed on the filter paper in each well, and the mites were incubated at 25°C in an incubator at a relative humidity of 75% (Nong *et al.*, 2014). Three replicates were performed for each concentration of stigmasterol extract. The viability of the mites was checked regularly by needle stimulation; mites that displayed no reaction were recorded as dead (Macchioni *et al.*, 2004). Fenvalerate only and distilled water and glycerin (1:1) were used as the positive control and negative control, respectively.

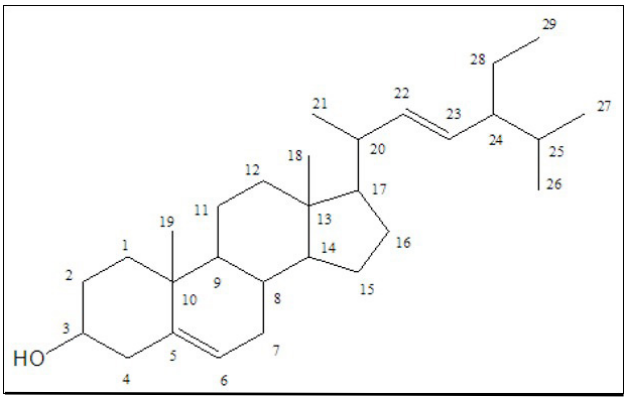

Fig. 1. The structure of stigmasterol.

RESULTS

Structure of stigmasterol

The compound was isolated as colorless needle crystal. The NMR spectra of the compound showed the presence of two double bonds (Table I) (δ_H 5.27, 1H, d, J = 4.8 Hz; δ_H 5.09, 1H, m; δ_H 5.00, 1H, m; δ_C 140.7 s; 138.3 d; 129.2 d; 121.7 d), two tertiary methyl (δ_H 0.68, 3H, s; δ_H 0.70, 3H, s), and one oxygenated carbon signal (δ_C 71.7 d). Therefore, the structure was identified as stigmasterol ($C_{29}H_{48}O$) (Fig. 1) based on comparisons of the spectroscopic data with that of data reported in literature (Yang *et al.*, 2006; Zhu *et al.*, 1997).

Insecticidal activity of stigmasterol

The stigmasterol compound was diluted for three experimental concentrations of 0.5%, 0.25%, and 0.125% (m/v). Each concentration of stigmasterol component was used to test the insecticidal activity. We observed the mite mortality of each group at 4, 12, and 24 h. Treatment with stigmasterol under 0.5% concentration could achieve 16.67% mite mortality within 12 h and 23.33% within 24 h. The insecticidal activity was poorer at concentrations below 0.5%.

Table I.- ^{1}H-NMR and ^{13}C-NMR data of stigmasterol (in $CDCl_3$), δ in ppm.

No.	^{1}H-NMR	^{13}C-NMR	No.	^{1}H-NMR	^{13}C-NMR
1		37.2	16		28.9
2		31.6	17		55.9
3	3.56 (m)	71.7	18	0.68 (s)	12.0
4		42.2	19	1.01 (s)	19.4
5		140.8	20		40.5
6	5.27 (d)	121.7	21		21.2
7		28.9	22	5.00 (m)	138.3
8		31.9	23	5.09 (m)	129.2
9		50.1	24		51.2
10		36.5	25		31.9
11		21.0	26		21.1
12		39.6	27		19.0
13		42.2	28		25.4
14		56.8	29		12.3
15		24.3			

The insecticidal activity of stigmasterol was weaker than that of ageraphorone compound obtained from *E. adenophorum* Spreng. through preliminary separation. However, in the experiments, we found an interesting phenomenon, that is, the dead state of mites treated with stigmasterol was similar to that of mites of the positive control group treated with fenvalerate.

Dead state of mites

Figure 2A shows the dead mites after treatment with stigmasterol. Figure 2B shows the natural dead state of mites from the blank control group while Figure 2C shows the dead state of mites in the positive control group after treatment with fenvalerate. The dead states of mites after treatment with stigmasterol and fenvalerate showed more normal, natural death.

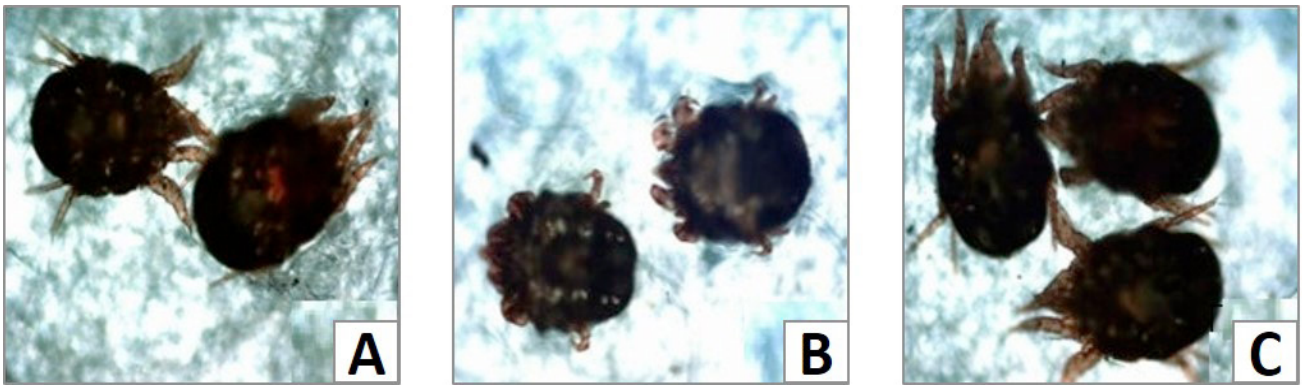

Fig. 2. Observations of the dead states of mites. **A,** treatment with stigmasterol; **B,** treatment with water and glycerin (1:1); **C,** treatment with fenvalerate.

In particular, no struggle and dehydration was observed. Therefore, the treatment of stigmasterol and fenvalerate showed an insecticidal activity. Meanwhile, the mites in Figure 2B showed a natural shrinking dead state. In particular, the mites curled and were unable to straighten legs, as well as narrow tail. Thus, the death of mites in Figure 2B was natural and was not due to the treatment of stigmasterol and fenvalerate.

DISCUSSION

E. adenophorum Spreng. contains many chemical components, such as alkaloids, sugar, sesquiterpenes, lipids, and coumarin materials. Several of these substances showed good insecticidal bioactivity. For example, the substance of alkaloids, namely, Eupatorin A, contains a strong biological activity against aphid (*Aphis gossypii*). In addition, 8aα-hydroxy-1-isopropyl-4, 7-dimethyl-1, 2, 3, 4, 6, 8a-hexahydro-naphthalene-2, 6-dione, a component extracted from *E. adenophorum* Spreng. leaves, showed good toxicity against ascariasis and antibacterial effect (He *et al*., 2006). Three ageraphorones, namely, 9-OXO-10, 11-dehydro-ageraphorone, 9-OXO-ageraphorone, and 9-β-hydroxy-ageraphorone, extracted from *E. adenophorum* Spreng. exhibited good biological activity against *P. cuniculi* (Nong *et al*., 2012, 2014). The separation and identification of the biological activity of these substances from *E. adenophorum* Spreng. can provide the basis for research on the medicinal value of this weed.

However, oral 9-OXO-10, 11-dehydro-ageraphorone can cause hepatotoxicity in mice (Bhardwaj *et al*., 2001; Guo *et al*., 2016). Currently, the evidence that ageraphorone is harmless to host animal is not yet solidly established. Hence, ageraphorone cannot be developed as an acaricidal agent. Therefore, nontoxic compounds should be isolated from *E. adenophorum* Spreng. extracts, and the development of nontoxic botanical insecticidal is particularly important. Our study showed that stigmasterol contains insecticidal activity against mites. In particular, the insecticidal activity of stigmasterol was weaker than that of ageraphorone compound obtained from *E. adenophorum* Spreng through preliminary separation. However, in the experiments, we found an interesting phenomenon, that is, the dead state of mites treated with stigmasterol was similar to that of mites of the positive control group treated with fenvalerate. This phenomenon is interesting and indicates that research on the insecticidal activity of stigmasterol has certain theoretical value. Stigmasterol is nontoxic but can be applied to cancer cells, and β-sitosterol and stigmasterol can induce the apoptosis of human liver cancer SMMC-7721 cells (Li *et al*., 2012). The stigmasterol from *Hedyotis diffusa* Willd. showed inhibitive effect on hepatoma cells in vitro and in vivo (Zhang *et al*., 2008). Stigmasterol insecticidal activity against *P. cuniculi* was similar with thymol, powdered form, with 3.2% oxalic acid (OA) against ectoparasitic mites, Varroa destructor Anderson (Rashid *et al*., 2012).

The good insecticidal activity of *E. adenophorum* Spreng. extracts has been proven. However, the present study is still nearly under the stage of using the crude extracts (Baruah *et al*., 1994). Our study results showed that stigmasterol contains insecticidal activity against mites and that the dead state of mites treated with stigmasterol was similar to that of mites of the positive control group treated with fenvalerate. We believe that this component can be developed as an effective pesticide through further research of its acaricidal activity mechanism.

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Statement of conflict of interest

The authors declare that they have no competing interests. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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