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Research Article

Effect of Pineapple Extracts on the Reproduction of *Rhipicephalus sanguineus* Semi-Engorged Females

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Abstract

Background and Objective: *Rhipicephalus sanguineus* (*R. sanguineus*), known as the brown dog tick, is a main vector of several important pathogens in dog and occasionally in humans. The present study investigated the *in vitro* effects of pineapple skin and core extracts on *Rhipicephalus sanguineus* semi-engorged females compared with a commercial chemical insecticide, in a search for new alternative methods to control this ectoparasite. **Materials and Methods:** A total of 180 female ticks with homogeneous weight were divided into four groups with three replications and each group was immersed for 5 min in the following solutions: 0.25% w/v fipronil (FIP), pineapple skin (PAS) extract, pineapple core (PAC) extract, or distilled water (CON) for the control group. After the immersion, the ticks were incubated at 28°C, RH>80% and a 12 h photoperiod for 14 days to analyze the toxicity and effects on morphological changes, mortality rates, laying rates and oocyte histological appearances. Data were analyzed using analysis of variance (one-way ANOVA). **Results:** The laying rates of female ticks in the treated groups (FIP 0%, PAC 26.70% and PAS 20.00%) were significantly lower than in the control group (46.70%) ($p<0.05$). The mortality rates in treated groups (PAC 100%, PAS and FIP 93.33%) were higher than in the control group (46.67%). For oocyte histology result, pineapple extracts had effect on structural changes in oocyte development which was similar to the effect of the chemical compound, including large vacuolated cytoplasm, size variations, laceration and withering of yolk granules. **Conclusion:** Extracts from the skin and core of pineapple can be considered as potent natural agents that can reduce the reproduction of *R. sanguineus* females and control tick population. However, the active ingredients and mechanism of this plant should be further clarified to improve the acaricidal efficiency.

Key words: *Rhipicephalus sanguineus*, pineapple, fipronil, mortality rate, laying rate, histology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rhipicephalus sanguineus known as the brown dog tick, is an important and widespread ectoparasite that primarily feeds on dogs[1,2]. It is a main vector of several important pathogens in veterinary medicine and occasionally in humans[3], e.g. *Ehrlichia canis*[4], *Babesia vogeli* and *Hepatozoon canis*[5] in canines and *Rickettsia rickettsii*[6], *Rickettsia conorii*[7] in humans. The pathogenicity in animals is due to blood loss during the tick feeding process, in addition to ticks being vectors of blood parasites which cause illness, anemia and nervous disorders and can lead to death[3,8-10].

There are two major ways to control tick infestation: chemical and non-chemical methods[2]. Fipronil, a commercial chemical insecticide, is currently a popular synthetic substance in the market which can be found in various forms such as spot-on and spray. This acaricide is a phenylpyrazole compound, which blocks transmission of signals by the inhibitory neurotransmitter GABA present in insects[11-13]. However, fipronil has side effects on animals and humans and is toxic to the environment as well as ectoparasites[13,14]. Due to these problems, a new alternative method of controlling ticks needs to be developed in order to reduce the harm from chemical compounds.

Pineapple (*Ananas comosus* L. Merrill) is widely grown in tropical and subtropical regions and is one of the important economic fruits in Thailand, which is the world's largest pineapple producer[15]. Bromelain, a mixture of cysteine protease, can be extracted from many parts of the pineapple, including the core, skin, stems, crown and leaves and is also extracted from pineapple waste during the production process[15-17]. The benefits of this proteolytic enzyme have been reported in a variety of medical and therapeutic applications, e.g., inhibiting the synthesis of fibrinogen[18], inhibiting platelet aggregation[19], promoting skin debridement[20], as an anti-inflammatory agent for osteoarthritis[21] and for its anti-cancer properties[22]. Extracts from various parts of the pineapple have shown antiparasitic activity on helminths such as *Haemonchus contortus*[23] and ectoparasites such as *Rhipicephalus (Boophilus) microplus*[24].

Thus, this study aimed to evaluate the *in vitro* effects of extracts from pineapple skin and core, which are a source of bromelain, compared with fipronil on morphological changes, mortality rate, laying rate and histology of the reproductive system of *R. sanguineus* semi-engorged females. The results of these experiments will hopefully promote further research into new alternative methods to control the tick population.

MATERIALS AND METHODS

This study was carried out at parasitology laboratory, Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, Thailand, from April-December, 2016.

Tick preparation: A total of 180 *R. sanguineus* semi-engorged females, average weight 28 mg, from one dog were used in this experiment. The ticks were separated into groups of 15, with three repetitions for each treatment, including fipronil, pineapple skin, pineapple core and control groups. Grouping modifications were suggested by Domingues *et al.*[24]. Before treatment, all ticks were photographed under a stereoscope (Nikon, Tokyo, Japan) for morphological observation.

Crude extract preparation: Pineapple extraction followed the method of Ketnawa *et al.*[25]. The pineapples (*Ananas comosus*) were purchased from a local market. The skin and core were removed and cut into small pieces, then blended using a juice blender (Tefal, Jakarta, Indonesia) for 3 min with cold distilled water in a proportion of 1:1. The material was filtered with a muslin cloth and centrifuged (Hettich, Kirchlengern, Germany) for 20 min at 10,000 rpm at 4°C. The supernatants were then collected as pineapple skin and core extracts. After preparation, the crude extracts were used in the experiment immediately.

Immersion test: *R. sanguineus* semi-engorged female ticks were washed with distilled water and then dried on absorbent paper. Afterwards, the ticks were immersed in petri dishes for 5 min following the method given by Domingues *et al.*[24]. The three treatment groups consisted of 0.25% w/v fipronil (FIP), pineapple skin extract (PAS), or pineapple core extract (PAC), while ticks in the control group (CON) were immersed in distilled water for the same time period[26]. The ticks were dried on absorbent paper and placed in an incubator (Memmert, Schwabach, Germany) under controlled conditions, 28 $\pm$ 1°C, RH>80% and a 12 h photo period, for 14 days. The acaricidal effect on semi-engorged ticks was investigated and recorded everyday of the experiment for calculation of mortality and laying rate. Dead ticks were identified by loss of motility and pedal reflex. After that, dead ticks were weighed and photographed under a stereoscope, followed by fixing in 10% formalin for histological examination. The calculations used in this experiment were:

$$\text{Mortality rate} = \frac{\text{Dead ticks}}{\text{Total number of ticks}} \times 100$$

$$\text{Laying rate} = \frac{\text{Laying ticks}}{\text{Total number of ticks}} \times 100$$

Histological study: To study the effects of chemical and natural compounds on the reproductive system of *R. sanguineus* semi-engorged females, the tegument of dead ticks was carefully perforated with fine needles, followed by fixing in 10% formalin[27]. After fixation, the ticks were processed according to routine histological techniques and embedded in paraffin. Each sample block was sectioned at a thickness of 4 µm using a rotary microtome (Leica, Wetzlar, Germany). Tissue sections of the ticks were stained with hematoxylin and eosin and the oocyte development observed under a light microscope (Olympus, Japan).

Statistical analysis: The body weight loss and laying rate were analyzed by one-way ANOVA (SPSS version 16.0, SPSS Inc., Chicago, IL., USA.). A $p \leq 0.05$ was considered statistically significant.

RESULTS

Morphological changes: The morphology of semi-engorged female brown dog ticks in all experiments changed after treatment. The changes included size, color and surface. At day 14 after treatment, ticks were constricted and decolorized. Ticks in the control group had a yellow-brown color and hard dry surface, but a glossy hard surface was found in the fipronil group. Dog ticks treated with pineapple extracts had yellow-black coloration and a soft surface (Fig. 1).

Mortality rate: Ticks were immersed in the various treatments and then incubated to study the mortality rate. The number of surviving ticks in each group was observed and recorded everyday post-treatment (Fig. 2). The first dead tick was found at day 6 after treatment in the PAS group, which was earlier than the other groups. Dead ticks were observed in the FIP and PAC groups at day 7 and the control group at day 8. At the end of the experiment, the highest mortality rate was observed in the PAC group (100%), followed by the PAS and FIP groups (93.33%). As expected, ticks in the control group had the lowest mortality rate (46.67%).

Laying rate: The effect of each treatment on the reproduction rate of *R. sanguineus* semi-engorged

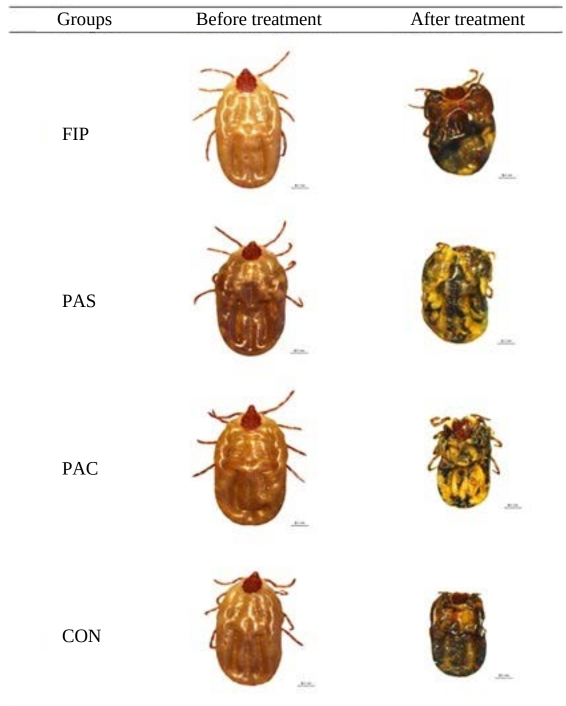

Fig. 1: Morphological comparison of *R. sanguineus* semi-engorged females from each treatment group. FIP: Treated with fipronil, PAS: Treated with pineapple skin extract, PAC: Treated with pineapple core extract, CON: Control. Bar = 0.1 cm

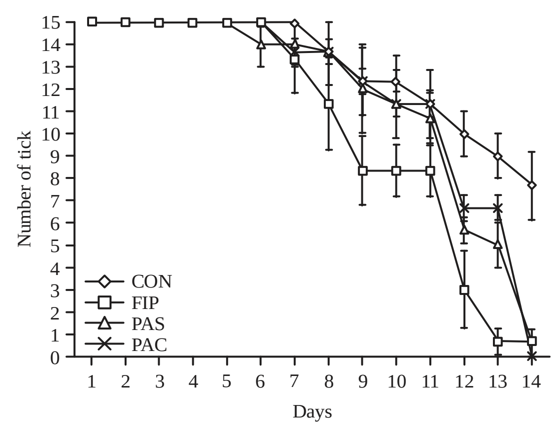

Fig. 2: Number of surviving *R. sanguineus* semi-engorged females in each treatment group during the experiment. CON: Control, FIP: Treated with fipronil, PAS: Treated with pineapple skin, PAC: Treated with pineapple core

females was examined by the laying rate, which was observed during incubation of ticks. This showed

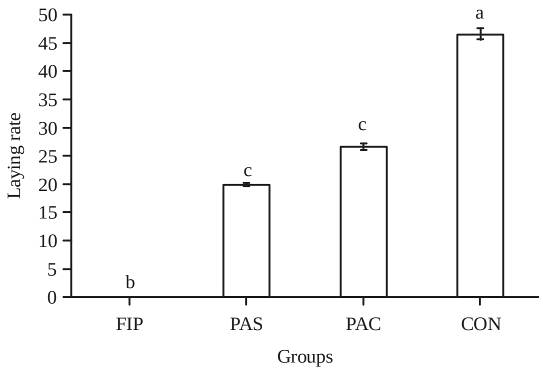

Fig. 3: Laying rates of *R. sanguineus* semi-engorged females. FIP: Treated with fipronil, PAS: Treated with pineapple skin, PAC: Treated with pineapple core, CON: Control. [a,b,c]Significant difference at $p<0.05$

that fipronil had the highest acaricidal activity, completely inhibiting oviposition of female ticks. Interestingly, the natural products (both skin and core of pineapple) had efficacy in controlling egg laying by ticks, although lower activity than the chemical product. The laying rates of female ticks treated with extracts of the skin and core of pineapple were 20.00 and 26.70%, respectively, significantly less ($p<0.05$) than the control group (46.70%) (Fig. 3).

Histology: In the present study, dead female ticks in all experiment were fixed and sectioned for investigation of acaricidal activity on the reproductive system. The developmental stages of oocytes of *R. sanguineus* in the control group were identified (Fig. 4a-e):

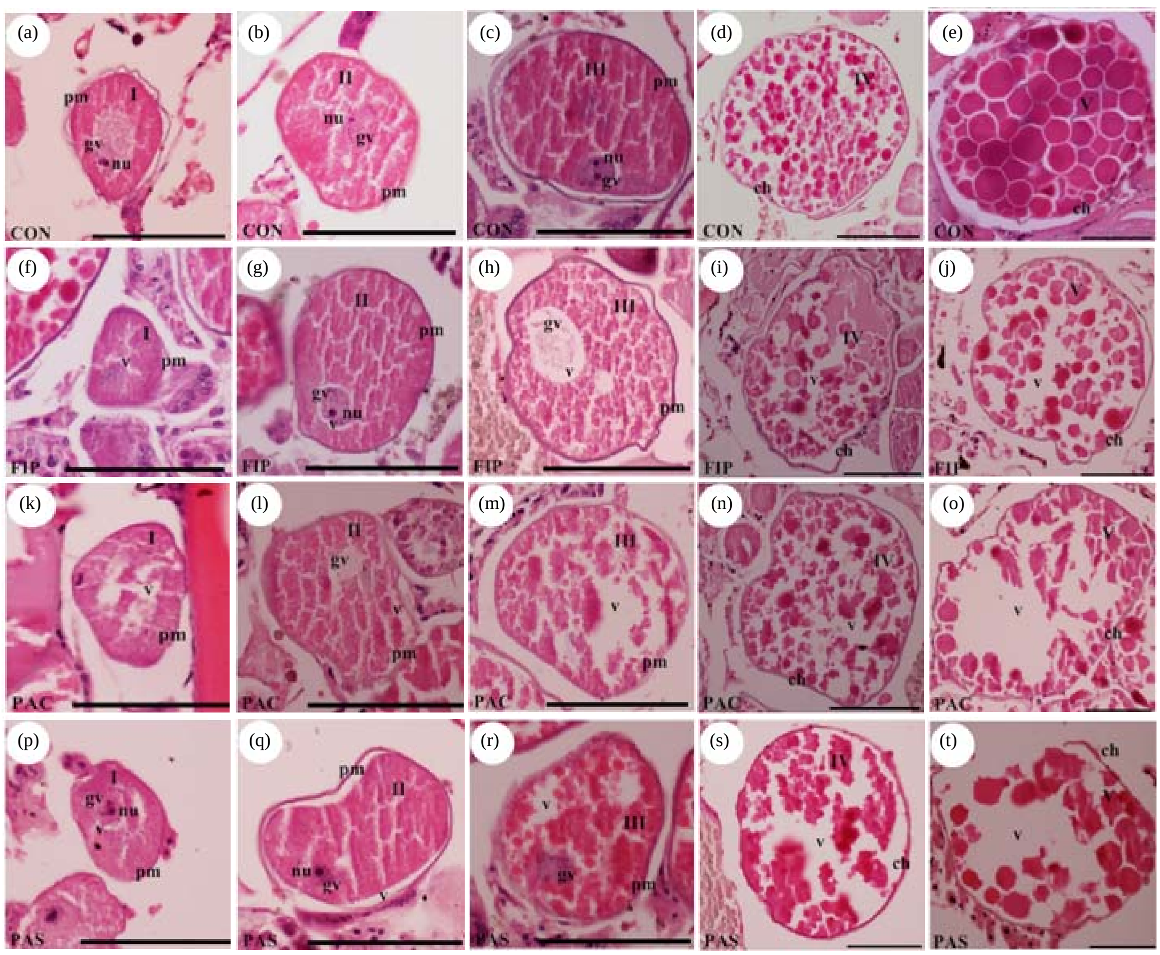

Fig. 4(a-t): Histological characteristics of *R. sanguineus* oocytes stained with hematoxylin and eosin. CON: Control, FIP: Treated with fipronil, PAC: Treated with pineapple core, PAS: Treated with pineapple skin, I: Oocyte stage I, II: Oocyte stage II, III: Oocyte stage III, IV: Oocyte stage IV, V: Oocyte stage V, pm: Plasma membrane, gv: Germ vesicle, nu: Nucleolus, v: Vacuole, ch: Chorion. Bar =100 μm. a-e: CON, f-j: FIP, k-o: PAC and p-t: PAS

- Oocytes I-had a clear germ vesicle with a nucleolus. The cytoplasm had a homogeneous appearance
- Oocytes II-had a germ vesicles in the center. The cytoplasm had a thin homogeneous granulation
- Oocytes III-were larger and plasmic membrane was thicker than oocyte I and II. The cytoplasm was full of yolk granules of various sizes
- Oocytes IV-were round and the germ vesicle was not observed due to the yolk granulation. The cytoplasm possessed countless yolk granules, in various sizes. The chorium was almost totally deposited
- Oocytes V-were the largest and presented a thick chorium. The cytoplasm was full of great yolk granules

Histological changes of the oocytes could be observed in all treatment groups. *R. sanguineus* semi-engorged females exposed to fipronil as a chemical acaricide showed histological variations of oocytes when compared with the control group. Hypochromatic germ vesicles surrounded by vacuoles were found in this treatment group (Fig.4f-h), while oocytes of late developmental stages IV and V displayed vacuolation in more than 50% of the cytoplasm area, including decreased yolk granules, with some rupturing and size variation (Fig. 4i, j).

The histological characteristics showed intensive change in both natural product groups, similar to the fipronil group (Fig. 4k-t). Especially severe alterations were revealed in stage IV and V oocytes, showing extensive vacuolation in the cytoplasm and fractured and withered yolk granules. Interestingly, pineapple skin extract caused laceration of the plasma membrane in stage V oocytes (Fig. 4t).

DISCUSSION

Rhipicephalus sanguineus, known as the brown dog tick, is an important ectoparasite of domestic dogs. A member of the family Ixodidae, it is the main vector of canine babesiosis, hepatozoonosis and ehrlichiosis and of tularemia in humans[28,29]. Currently, one of the most efficient methods of controlling ticks is the use of chemical products or acaricides, which interfere with the nervous system and inhibit the development and reproduction of ticks. Several studies have demonstrated the effectiveness of these synthetic compounds ectoparasites, but their mechanism of action on the ovaries of these ecto parasites remains unclear.

Fipronil is a highly effective and widely used acaricide belonging to the Phenylpyrazole family[30]. It is highly toxic to insects, disrupting the central nervous system by blocking the GABA-gated[11-13]. Thus, fipronil is popular for use in tick management in dogs. In the present experiment, fipronil was effective against brown dog ticks by killing and entirely inhibiting oviposition for 14 days after treatment, in accordance with a previous report[31]. Moreover, oocytes of *R. sanguineus* semi-engorged females exposed to fipronil exhibited abnormal histological characteristics, developmental stages including size changes, vacuolization in the cytoplasm, rupture of yolk granules and yolk granules of various sizes. This result indicated that fipronil also has an impacton the vitellogenesis of *R. sanguineus* semi-engorged females, leading to control of the population of this ectoparasite[31,32]. However, fipronil has been found to induce hepatotoxicity in animals and humans[13] and also has been classified as a possible human carcinogen[33,34]. Therefore, alternative ways have been developed to control ectoparasites that are safe for the environment, animals and human health.

The present study collected the skin and core of pineapples, which are considered waste materials, to investigate their acaricidal activity on the reproduction of *R. sanguineus* semi-engorged females. Pineapple is an important source of cysteine proteinase enzyme and bromelain. These enzymes can be extracted from many parts of the pineapple, such as the stem and peel[24,25,35]. Bromelain has proteolytic activity that can break the peptide bonds of the protein structure[24,36]. The proteolytic properties of this enzyme have been extensively researched, especially in medical studies including inhibition of platelet aggregation[29,37], anti-inflammatory[21,35,37-39] and anti-cancer properties[38,40], induction of wound healing and skin debridement[20,37,41] and anthelminthic activity against cow ticks[23,24,42-45]. This is the first report on the activity of pineapple extract on vitellogenesis of *R. sanguineus* semi-engorged females. The pineapple skin and core extracts revealed efficacy in inhibiting the reproductive system of female brown dog ticks by disrupting oocyte development, with results similar to fipronil. Severe histological alterations, size variation, laceration and withering of yolk granules were observed in the late stages of vitellogenesis (oocytes III-V). In this experiment, drastic oocyte damage, including cytoplasmic vacuolation, was observed in both extract groups. According to Carvalho and Recco-Pimentel[46] and Junqueira and Carneiro[47] the presence of autophagic vacuoles in the cells is caused by the necessity to degrade or recycle portions of the cytoplasm, such as modified or damaged organelles. The purpose of this is to reabsorb some mass and/or the remaining cell portions and to reutilize components of the cell. Therefore, the increasing amount and size of these vacuoles in oocytes indicated that both extracts were highly toxic and caused damage to the cell. The histological appearance of the treatment groups was correlated with the laying rate results, which showed that the pineapple skin and core extracts significantly inhibited oviposition compared with the control group.

CONCLUSION

These results demonstrated that pineapple extracts affected the mortality and vitellogenesis of *R. sanguineus* semi-engorged females, effects similar to those of fipronil, indicating that these plant extracts could be promising acaricides. The use of pineapple waste extracts is an inexpensive strategy that is less toxic to the hosts and has a low impact on the environment. Further studies are needed to clarify the enzyme activity and mechanism of bromelain on tick reproduction in order to develop natural products as alternative methods for tick population management.

SIGNIFICANCE STATEMENT

This study discovered that the effects of pineapple skin and core extract affecting the mortality and vitellogenesis of *R. sanguineus* semi-engorged females, effects similar to those of fipronil. Thus, pineapple crude extract, which is a natural compound, can be used to control *R. sanguineus* in dogs. Therefore, the enzyme activity and mechanisms of pineapple extract on tick reproduction for the control of tick population should be clarified in further studies.

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