EFFECTS OF ETHANOLIC EXTRACT FROM PINEAPPLE (*ANANAS COMOSUS*) PEEL ON *RHIPICEPHALUS SANGUINEUS* S. L. (ACARI: IXODIDAE)

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Abstract. Canine hemoparasitic infections, such as anaplasmosis, babesiosis, ehrlichiosis, and hepatozoonosis, are worldwide life-threatening tick-borne diseases. Main tick species carrying the infective agents in Thailand is *Rhipicephalus sanguineus* s. l., or the brown dog tick. Various types of chemical substances, as well as plant ingredients, have been used for tick control, but development of resistance to anti-tick synthetic chemicals poses problems. Ethanolic extract of pineapple peel (PPEE) and a dog shampoo formulation composed of PPEE (DSPP) were tested against male and female (non- and engorged) *R. sanguineus*. Compared to 6% (w/v) flumethrin aqueous solution all three PPEE solutions [25%, 50% and 75% (w/v)] exhibited as good of better immobilization effect against engorged and non-engorged ticks, and the two higher PPEE concentrations were lethal (26-53%) to engorged ticks and all three concentrations to non-engorged ticks (33-53%) after 24 hours of immersion. DSPP preparations [1.5%, 2.5% and 3.5% (w/v)] were equally effective in immobilizing engorged and non-engorged ticks as a commercial permethrin [0.5% (w/v)] dog shampoo (DSP), but all dog shampoos were not lethal to engorged ticks, except DSPP at the highest concentration to a small extent (7%) while the three DSPP preparations were more lethal (18-28%) to non-engorged ticks than DSP (15%) after 24 hours of treatment. Thus, aqueous solutions and dog shampoo preparations of pineapple peel ethanol extract should be considered as alternative options to chemical acaricides for controlling brown dog ticks.

Keywords: *Ananas comosus*, *Rhipicephalus sanguineus* s. l., dog shampoo, ethanolic extract, pineapple peel

INTRODUCTION

Rhipicephalus sanguineus s. l. (brown dog tick), an ordinary ectoparasite of domestic dogs, is distributed almost worldwide in tropical and subtropical areas (Dantas-Torres, 2008; 2010; Gray *et al*, 2013; Dantas-Torres *et al*, 2018). *R. sanguineus* s.l. is the vector of several tick-borne animal pathogens, such as *Anaplasma platys*, *Babesia canis vogeli*, *Candidatus* Mycoplasma haematoparvum, *Ehrlichia canis*, and *Hepatozoon canis* (Liu *et al*, 2016;

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Kaewmongkol *et al*, 2017), as well as human pathogens, such as *Rickettsia* spp (Nicholson *et al*, 2010; Álvarez-Hernández *et al*, 2017).

Various synthetic chemical agents, such as amitraz, fipronil, macrocyclic lactones, and permethrin, are used as an acaricide for controlling tick infestation (Piesman, 2006; Nolan and Lok, 2012). However, intensive use of synthetic acaricides can lead to drug resistance resulting in treatment failure (Abbas *et al*, 2014; Rodriguez-Vivas *et al*, 2018), in addition to posing potential risks to human health and of environmental pollution (Dantas-Torres, 2008; Eiden *et al*, 2015) as well as imposing economic loss to the pharmaceutical industry involved in their production (Graf *et al*, 2004). Amitraz, coumaphos, dichlorodiphenyltrichloroethane, and permethrin failed to control ticks in Panama (Miller *et al*, 2001), and, more recently, fipronil and permethrin in USA (Eiden *et al*, 2015) and amitraz and cypermethrin in Mexico (Rodriguez-Vivas *et al*, 2017). Brown dog ticks resistant to ivermectin have been reported (Fernández-Salas *et al*, 2012; Rodriguez-Vivas *et al*, 2017). Thus, there is an urgent need to find alternative sources of acaricides and to develop vaccines.

In tropical and sub-tropical regions, fruit extracts have shown acaricidal effects. The ethanol and hexane extracts of chinaberry (*Melia azedarach*) ripe fruits cause lethality of cattle tick *R.* (*Boophilus*) *microplus* larvae and have a partial effect on egg production and embryogenesis of engorged ticks (Borges *et al*, 2003), while green fruit hexane extract affects *R. microplus* oogenesis (de Sousa *et al*, 2013). Aqueous extract of sugar-apple (*Annona squamosa*) fruit peel together with 80% neem (*Azadirachta indica*) seed oil exerts 100% larvicidal activity against *R. microplus* larvae after a 2-hour treatment (Madhumitha *et al*, 2012). Methanol extracts of black pepper (*Piper nigrum*) and Indian long pepper (*P. longum*) manifest acaricidal and oviposition effects on adult *R. microplus* (Godara *et al*, 2018).

Pineapple (*Ananas comosus*) by-products or wastes, such as pineapple peel (PP), have been used in cattle feed or as fertilizer, but large amounts are still discarded daily, especially from fruit juice and canning factories (Ketnawa *et al*, 2011; Sukruansuwan and Napathorn, 2018). Aqueous extract and bromelain from PP inhibit hatching and oviposition of engorged female *R. microplus* (Domingues *et al*, 2013). Recently, distilled water extracts of PP and core were shown to be effective against female semi-engorged *R. sanguineus* s. l. oocyte development, egg laying and longevity (Juasook *et al*, 2018). However, there is a lack of information regarding the effects of PP extracts and formulated shampoo on male and non-engorged with distilled water (1:1 ratio) before used. Hence, the effects of pineapple peel ethanolic extract (PPEE) and dog shampoo preparation (DSPP) against adult *R. sanguineus* were investigated. Findings should assist in future development of PP as a safe and inexpensive acaricide against brown dog ticks and provide added value to waste from pineapple industry in Thailand.

MATERIALS AND METHODS

Sample collection

Adult male and female *R. sanguineus* ticks were collected from 10 dogs in Bangkok metropolis and 25 dogs in Nakhon Pathom Province, Thailand. Dogs (nine male and 26 female mongrels 1-8 years of age) were not treated with anti-ectoparasitic agents for at least three months prior to tick collection. Ticks were randomly collected from five skin areas (right and left ears, neck, lumbar region, and tail) and separated into engorged (female, *n* = 405) and non-engorged (male, *n* = 675 and female, *n* = 675) specimens, washed with distilled water and kept at ambient temperature for three days prior to experimentation. Species and gender were determined under a stereomicroscope (Nikon SMZ 745, Tokyo, Japan), 50x magnification, based on key structures, such as basis capituli, dorsal scutum and male adanal plates (Dantas - Torres *et al*, 2013).

Study protocol was approved by the Faculty of Veterinary Science-Animal Care and Use Committee, Mahidol University (MUVS-2015-40).

Plant extraction

Pineapple (*A. comosus* L. Merr var. Smooth cayenne) peel was obtained from a local market in Chon Buri Province, Thailand. Fresh PP was immediately dried in a hot air oven at 70°C for 72 hours, then coarsely ground using a high speed grinder NT-1000D (Nanotech, Bangkok, Thailand) and extracted with 95% ethanol (1 kg of dried peel per 1.5 l of ethanol) in the dark at ambient temperature for 5 days before being filtered through a Whatman no. 41 filter paper (Whatman, Pittsburgh, PA). Filtrate was concentrated in a rotary evaporator at 40°C [Rotavapor R-205; Büchi (Thailand), Bangkok, Thailand], then evaporated to dryness using a freeze-dryer (FreeZone Freeze Dryer; Labconco, Kansas City, MO) for 24 hours and stored at -20°C until used within one month.

Tick immersion assay

Engorged and non-engorged ticks were divided equally in each treatment group. One engorged tick and three non-engorged ticks in separate wells of a 24-well culture plate were immersed in 3 ml of various concentrations of PPEE for 5 minutes, washed with distilled water and dried on a towel paper. Controls were ticks treated with 6% (w/v) flumethrin (Bayer Thai, Bangkok, Thailand) and then with distilled water. Each treated tick was observed under a stereomicroscope, at 7x or 50x magnification, every 5 minutes for 24 hours for movement normal, immobile (no movement of legs and body for at least two consecutive observations) or dead (no response to a tactile stimulus, a CO_2 stimulus or blown air on six consecutive occasions). Each experiment was conducted in triplicate.

PP ethanolic extract dog shampoo formulation (DSPP)

DSPP was prepared by mixing PP ethanolic extract [3%, 5% and 7% (w/v)] with a solution of 1,3-dimethylol-5,5-dimethylhydantoin (Sigma-Aldrich, St. Louis, MO), sodium lauryl ether sulfate (Sigma-Aldrich), sodium chloride (Sigma-Aldrich), cocamidopropyl betaine (Sigma-Aldrich), and coconut diethanolamine (Chemipan, Bangkok, Thailand), in distilled water, pH 6.5-7.5. Positive and negative control was 1% (w/v) permethrin dog shampoo (DSP) (Sherwood Chemicals, Bangkok, Thailand) and solvent used in DSPP, respectively. All shampoo preparations were diluted 1:2 with distilled water before used in the tick immersion assay as described above.

Statistical analysis

Number of affected ticks in each treatment group is reported as mean percentage ± SD from experiment conducted in triplicate. Chi-square test was used to compare non-parametric data using SPSS 24.0 program (BMI, Armonk, NY), with statistical significance accepted at *p*-value <0.05.

RESULTS

Effects of PP ethanolic extracts (PPEE) on engorged and non-engorged *R. sanguineus* ticks

Immobilization of (female) engorged ticks by immersion with distilled water was apparent at 15 minutes and then increased at minute 30 and remained unchanged till end of experimental period (Table 1). At minute 15 of treatment all three PPEE and flumethrin solutions resulted in significantly increased immobilization of engorged ticks compared to distilled water control. The effect of flumethrin increased at minute 30 and remained unchanged throughout the remaining course of treatment. Immobilization ability of PPEE solutions are significantly higher than flumethrin at minute 15; for 25% (w/v) PPEE solution, the effect decreased continuously till hour 1 and then remained constant, but lower than flumethrin, till hour 24; the effects from immersion in 50% (w/v) PPEE solution fluctuated during the following 11.5 hours, then dropped drastically at hour 24; and treatment with 75% (w/v) PPEE solution decreased at minute 30 and remained constant, but higher than flumethrin, during the following 11.5 hours (with a slight drop at hour 3) before demonstrating a marked decrease at hour 24 (Table 1). The low percent immobilized engorged ticks observed at hour 24 of immersion with 50% and 75% (w/v) PPEE solutions was due to presence of dead ticks, which were only apparent at these two occasions (Table 2).

Table 1
Percent immobilized engorged female and non-engorged female and male *Rhipicephalus sanguineus* ticks following immersion in pineapple peel ethanolic extract (PPEE) and flumethrin solutions.

Treatment/ immersion period	Percent ± SD immobilized engorged ticks in treatment group (n = 27)					
	15 minutes	30 minutes	1 hour	3 hours	12 hours	24 hours
Distilled water	11 ± 0.8	30 ± 1.3	30 ± 0.9	30 ± 2.5	30 ± 0.5	22 ± 2.4
Flumethrin[a]	63 ± 1.7*	85 ± 0.5*	85 ± 1.7*	85 ± 3.7*	85 ± 0.5*	85 ± 0.9*
PPEE25[b]	96 ± 0.9*#	93 ± 0.7*#	70 ± 0.8*#	74 ± 0.5*#	70 ± 0.9*#	78 ± 1.5*#
PPEE50[c]	89 ± 1.1*#	96 ± 1.0*#	78 ± 1.8*#	93 ± 2.5*#	78 ± 2.1*#	63 ± 0.3*#
PPEE75[d]	100 ± 0.0*#	93 ± 0.6*#	93 ± 1.2*#	74 ± 0.3*#	93 ± 1.1*#	37 ± 0.6*#
	Percent ± SD immobilized non-engorged ticks in treatment group (n = 90)					
Distilled water	68 ± 1.2	78 ± 0.9	88 ± 1.6	80 ± 1.0	73 ± 1.3	82 ± 0.8
Flumethrin[a]	93 ± 0.7*	98 ± 0.4*	97 ± 0.1*	82 ± 0.7	72 ± 0.8	60 ± 0.3*
PPEE25[b]	97 ± 0.5*	98 ± 0.5*	97 ± 0.4*	78 ± 0.9	68 ± 0.4*	63 ± 0.2*
PPEE50[c]	97 ± 0.2*	99 ± 0.3*	94 ± 0.4*	70 ± 0.5*#	57 ± 0.6*#	54 ± 0.5*#
PPEE75[d]	100 ± 0.0*	98 ± 0.3*	92 ± 0.5*	63 ± 0.3*#	49 ± 0.3*#	45 ± 0.5*#

[a]6% (w/v). [b]25% (w/v). [c]50% (w/v). [d]75% (w/v). *p-value <0.05 compared to distilled water treatment, chi-square test. #p-value <0.05 compared to fluometuron treatment, chi-square test. Each experiment was conducted in triplicate.

Non-engorged (female and male) ticks were more sensitive to immobilization by all test solutions, including distilled water, compared to engorged ectoparasites (Table 1). Treatment with PPEE and flumethrin solutions elicited higher effects at all time points compared to distilled water. Immobilization was nearly 100% by immersion in the three PPEE and flumethrin solutions, then decreased from hour 3 to hour 24 of treatment, the percent immobilized ticks being comparable between flumethrin and 25% (w/v), but lower when compared to 50% and 75% (w/v) PPEE preparation, these phenomena owing to the presence of dead ticks (Table 2). In each treatment group male and female ticks were equally affected.

Effects of DSPP and DSP (dog shampoos) on engorged and non-engorged *R. sanguineus* ticks

Immersion with DSPPs [1.5%, 2.5% and 3.5% (w/v)] immobilized almost all engorged ticks throughout the experimental period. The drop in percent immobilized ticks at the later time points could be due to presence of dead ticks although definitive observation of such ticks was after 24 hours immersion in 3.5% (w/v) DSPP (Table 3). DSP was equally effective (with some fluctuations), but it is worth noting that solvent exerted a significantly high immobilization effect. As regards non-engorged ticks, solvent is significantly similarly effective and consistent in immobilization (with some fluctuations) throughout the treatment duration (Table 3). At early time points, there are no differences in percent immobilized ticks among 1.5% (w/v) DSPP, DSP and solvent treatments, but the dog shampoos are significantly more effective than solvent at hour 12 onwards, with DSPP preparation being more effective than DSP. Significant immobilization was observed with 2.5% and 3.5% (w/v) DSPP preparations at minute 30 and remained relatively constant till the completion of the experiment, except for a drop at hour 24 in the 3.5% (w/v) DSPP treatment group, owing to the presence of dead ticks, although such ticks were observed

Table 2
Percent ± SD dead engorged (E) and non-engorged (NE) *Rhipicephalus sanguineus* ticks following immersion in pineapple peel ethanolic extract (PPEE) and flumethrin solutions.

Treatment/ period	Percent ± SD dead ticks in treatment group (E, n = 27; NE, n = 90)							
	1 hour		3 hours		12 hours		24 hours	
	E	NE	E	NE	E	NE	E	NE
Distilled water	-	-	-	-	-	-	-	-
Flumethrin[a]	-	-	-	14 ± 0.6*	-	26 ± 1.0*	-	37 ± 0.1*
PPEE25[b]	-	1 ± 0.3*#	-	5 ± 0.3*#	-	30 ± 0.5*	-	33 ± 0.5*
PPEE50[c]	-	4 ± 0.3*#	-	29 ± 0.8*#	-	43 ± 0.1*#	26 ± 0.1*#	45 ± 0.7*#
PPEE75[d]	-	7 ± 0.6*#	-	37 ± 1.1*#	-	50 ± 0.5*#	52 ± 0.1*#	53 ± 0.4*#

[a]6% (w/v). [b]25% (w/v). [c]50% (w/v). [d]75% (w/v). #p-value <0.05 compared to flumethrin treatment, chi-square test. Each experiment was conducted in triplicate.

Table 3
Percent immobilized engorged female and non-engorged female and male *Rhipicephalus sanguineus* ticks following immersion in pineapple peel ethanolic extract (DSPP) and permethrin (DSP) dog shampoos.

Treatment/ period	Percent ± SD immobilized engorged ticks in treatment group (n = 27)					
	15 minutes	30 minutes	1 hour	3 hours	12 hours	24 hours
Solvent[a]	81 ± 0.5	74 ± 0.5	98 ± 0.4	70 ± 0.5	70 ± 0.1	97 ± 0.8
DSP[b]	81 ± 0.6	98 ± 0.2*	97 ± 0.2	67 ± 0.3	98 ± 0.5	67 ± 0.2*
DSPP1.5[c]	98 ± 0.2*	98 ± 0.4*	99 ± 0.1	97 ± 0.4*#	98 ± 0.4	98 ± 0.3#
DSPP2.5[d]	99 ± 0.8*	98 ± 0.4*	96 ± 0.4	98 ± 0.1*#	74 ± 1.4#	63 ± 0.4*
DSPP3.5[e]	98 ± 0.2*	96 ± 0.8*	98 ± 0.4	97 ± 0.6*#	98 ± 0.2*	67 ± 0.9*
	Percent ± SD immobilized non-engorged ticks in treatment group (n = 90)					
Solvent[a]	65 ± 0.9	57 ± 0.6	68 ± 0.1	92 ± 1.4	73 ± 2.2	69 ± 0.8
DSP[b]	63 ± 0.2	58 ± 1.1	77 ± 0.8*	91 ± 0.6	86 ± 0.5*	70 ± 1.3
DSPP1.5[c]	67 ± 0.2	58 ± 1.1	77 ± 0.3*	91 ± 0.5	96 ± 0.9*#	79 ± 0.1*#
DSPP2.5[d]	67 ± 0.5	81 ± 0.5*#	82 ± 0.8*#	84 ± 0.5	80 ± 0.9*#	78 ± 1.2*#
DSPP3.5[e]	78 ± 1.3*#	93 ± 0.3*#	84 ± 0.3*#	90 ± 2.0	79 ± 0.6*#	68 ± 2.6

[a]Used in DSP. [b]0.5% (w/v). [c]1.5% (w/v). [d]2.5% (w/v). [e]3.5% (w/v). *p-value <0.05 compared to solvent treatment, chi-square test. #p-value <0.05 compared to DSP treatment, chi-square test. Each experiment was conducted in triplicate.

at lower amounts at hour 12 in both 2.5% and 3.5% (w/v) DSPP treatment groups (Table 4). After 24 hours of immersion, all three DSPP preparations exhibited greater lethality in a dose-dependent manner than DSP. Both female and male ticks were equally affected.

DISCUSSION

Effective control of ticks infesting canine hosts and residing in the environment requires an integrated control strategy. Control methods should be performed using both protective measures of host (repellent, protective garment and tick removal) and tick management (Stafford *et al*, 2017). Various ectoparasiticides and repellents composed of chemical substances, such as spot-on formulations, chewable tablets, impregnated collars, shampoos, sprays, dips, and powders, are commercially available worldwide, with synthetic pyrethroids, flumethrin and permethrin, being most commonly used on domestic animals and in residential environment.

Our results reveal adult engorged *R. sanguineus* ticks are resistant to the lethal acaricidal effects of flumethrin solution [6% w/v)] and permethrin dog shampoo [0.5% (w/v)] over a 24-hour immersion period, but the latter was better in an immobilization assay. Ticks highly resistant to flumethrin and permethrin carry mutation in *para*-sodium channel (Miller *et al*, 2001; Jonsson *et al*, 2010; Tucker *et al*, 2017), the target of these acaricides (Vais *et al*, 2001). These findings suggest *R. sanguineus*

Table 4
Percent dead engorged (E) and non-engorged (NE) *Rhipicephalus sanguineus* ticks following immersion in pineapple peel ethanolic extract (DSPP) and permethrin (DSP) dog shampoos.

Treatment/ period	Percent ± SD dead ticks in treatment group (E, *n* = 27; NE, *n* = 90)					
	6 hours		12 hours		24 hours	
	E	NE	E	NE	E	NE
Solvent[a]	-	-	-	-	-	-
DSP[b]	-	-	-	2 ± 0.9*	-	15 ± 0.5*
DSPP1.5[c]	-	-	-	-	-	18 ± 1.0*
DSPP2.5[d]	-	1 ± 0.1*#	-	10 ± 0.9*#	-	22 ± 1.9*#
DSPP3.5[e]	-	4 ± 0.9*#	-	11 ± 0.5*#	7 ± 0.5*#	28 ± 1.0*#

[a]Used in DSP. [b]0.5% (w/v). [c]1.5% (w/v). [d]2.5% (w/v). [e]3.5% (w/v). *p-value <0.05 compared to solvent treatment, chi-square test. #p-value <0.05 compared to DSP treatment, chi-square test. Each experiment was conducted in triplicate.

ticks removed from dogs in Bangkok metropolis and Nakhon Pathom Province, are already resistant to flumethrin and permethrin.

Plants are a source of diversity for phytochemical compounds, which may provide acaricidal properties with less toxicity toward the environment and humans. Research on effective and environmentally-friendly control of ticks, as a strategy to replace synthetic acaricides, is an important challenge. In the present study, DSPP preparations [1.5%, 2.5% and 3.5% (w/v)] were more effective, particularly at the higher concentrations, at immobilizing and killing engorged and in particular non-engorged ticks compared to DSP [0.5% (w/v)]. These observations also applied to aqueous solutions of PPEE [25%, 50% and 75% (w/v)] compared to flumethrin [6% (w/v)]. Ethanolic extracts of pineapple peel and core have previously been reported to affect vitellogenesis and be lethal to semi-engorged *R. sanguineus* s. l. (Juasook *et al*, 2018) as well as of pineapple peel aqueous extract on engorged *R. microplus* (Domingues *et al*, 2013).

The proposed major active PPEE compound is bromelain, a cysteine proteinase (Ketnawa *et al*, 2011). Bromelain also affects nematodes, such as *Ancylostoma ceylanicum* and *Rodentolepis microstoma*, by damaging cuticles and parasite surface, decreasing motility and ultimately causing death (Stepek *et al*, 2007a). Furthermore, *Trichuris muris* (Stepek *et al*, 2006), *Protospirura muricola* (Stepek *et al*, 2007b) and *Heligmosomoides polygyrus* (Stepek *et al*, 2007c) show rapid decline in motility following bromelain treatment. Tick cuticle is composed of wax, lipid and protein matrix (Kaufman and Flynn, 2018) similar to that of nematodes (Page *et al*, 2014), accounting for the action of pineapple bromelain. The pronounced effect of dog shampoo solvent on tick immobilization could be attributed to the detergent components that are able to dissolve wax and lipids of the ectoparasite surface.

The weakness of the study was the heterogeneity of the tick life stages and age, which would vary in composition for each lot of ticks tested. As tick biological age is correlated with amount of surface cuticle (Balashov *et al*, 2009; Flynn and Kaufman 2015), thereby accounting for the fluctuations in the observed results. Additionally, determination of tick immobilized and death status is highly subjective. A more objective and quantitative assay is needed as well as uniform sets of ticks, if future studies are to involve comparisons of extracts of various types of plants and different extracting solvents.

In summary, the present work confirms the effectiveness of pineapple peel ethanol extract as an acaricidal agent, both as an aqueous solution or a dog shampoo, comparable to or better than the currently used synthetic acaricides flumethrin and permethrin. Further studies should investigate the active components of pineapple peel ethanol extract, efficacy of other types of organic extracting solvents and optimization of the shampoo solvent mixture. Development of acaricides from plant natural products will reduce the dependence on synthetic compounds with their inherent hazards to human health and the environment, and assist in giving added value to agricultural (fruit) waste materials.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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