

Full Length Research Paper

Pathophysiological effects of Neem (*Azadirachta indica*) derivatives to *Rhipicephalus appendiculatus*, the tick vector for Theileriosis (East Coast Fever)

Godwin P. Kaaya¹ and Marius Hedimbi^{2*}

¹School of Biological Sciences, University of Nairobi, P. O. Box 30197-00100, Nairobi, Kenya.

²School of Medicine, Department of Biochemistry and Microbiology, University of Namibia, P. O. Box 13301, Windhoek, Namibia.

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The effects of 2 Neem derivatives, Neem oil (NO) and Neem Seed Powder (NSP), on various physiological parameters of the brown ear tick, *Rhipicephalus appendiculatus*, the vector for *Theileria parva*. Neem oil was applied on rabbit ears using a fine brush, whereas NSP was mixed with rabbit pellets at various concentrations and fed on goats on which various instars of the tick were allowed to feed. Tick larvae smeared with Neem oil (NO) while attached to rabbit ears exhibited significant mortalities. The 10% NO induced a mortality of 40% compared to less than 1% observed in Peanut oil (PO) control. Furthermore, engorgement weights were significantly reduced (0.1 mg NO vs 4 mg PO). Their corresponding moulting percentages were 1 and 71% in NO and PO, respectively. Adult female ticks exhibited reduced engorgement weights and egg mass. Larvae able to attach on goats maintained on 100% NSP were only 11% compared to 66% for control goats fed on rabbit pellets (RP). The corresponding nymphal attachments were 24% NSP and 79% RP. The number and weights of eggs produced by adult ticks fed on goats maintained on NSP also dropped significantly. The hatchability of eggs produced by these ticks was also significantly reduced.

Key words: East Coast Fever, economic losses, Neem derivatives, *Rhipicephalus appendiculatus*, *Theileria parva*, tick control.

INTRODUCTION

East coast fever (ECF) is a cattle disease caused by the protozoan parasite, *Theileria parva*, transmitted by the brown ear tick, *Rhipicephalus appendiculatus* (Norval et al., 1992). It is the most important disease of cattle in 11

countries in eastern, central and southern Africa, namely; Kenya, Uganda, Tanzania, Burundi, Rwanda, Malawi, Mozambique, South Sudan, Democratic Republic of Congo, Zambia and Zimbabwe (Lawrence

*Corresponding author. E-mail: mhedimbi@unam.na.

et al., 1992; Laisser et al., 2014). About 28 million cattle in these regions are at risk from ECF (Mukhebi et al., 1992; Laisser et al., 2014) and annual mortality is as high as 40 to 80% among unvaccinated zebu calves (Homewood et al., 2006; Kivaria et al., 2007; Laisser et al., 2014). In Kenya, ECF has been reported to kill about 100,000 cattle of all ages annually and is believed to be the most important cattle disease in the country (Mbogo et al., 1995). The overall economic losses due to ECF in the endemic region has been estimated at US\$ 315 million annually (Minjauw and McLeod, 2003). Economic losses can be classified as direct e.g. mortality and milk loss and as indirect losses e.g. cost of controlling the disease, cost of research, training and extension services (Mukhebi, 1992; Kivaria et al., 2007). Furthermore, ECF is a major hindrance to the introduction of improved exotic breeds due to their high susceptibility to ECF (Minjauw and McLeod, 2003), a loss referred to as “lost potential” (Gachohi et al., 2012).

The control of ECF relies heavily on chemical acaricides applied on animals by plunge dips or spraying to kill the vector tick. The problems associated with this method include the development of resistance, contamination of meat and milk with toxic residues, environmental pollution and high production costs (Jonsson, 1997; Hedimbi et al., 2011). Ticks easily develop resistance to these chemicals (Wharton, 1976; Hedimbi et al., 2011). For instance, in Australia and South Africa, resistance to benzene hexachloride in ticks developed within 18 months after its introduction in the market. Furthermore, in South Africa, resistance to toxaphene and dichlorodiphenyltrichloroethane (DDT) developed after only 4 and 5 years, respectively (Wharton and Roulston, 1970; Kaaya, 1992). Since acaricide application is a continuous activity, it is expensive and difficult to sustain. The rate at which ticks are developing resistance coupled with the high costs of developing new chemical acaricides therefore, calls for alternative methods of tick control that are environmentally safer, cost-effective and sustainable (Sutherst, 2001; Polar et al., 2008).

Numerous studies on African plants with potential for control of *R. appendiculatus* have been undertaken. For instance, Maradufu (1982) reported that a hexane soluble viscous oil extract from the gum of *Commiphora myrrh* was repellent to adult *R. appendiculatus*. Three furanosesquiterpenoids isolated from a related African plant, *Commiphora erythraea* were also found to be toxic to larvae of *R. appendiculatus* (Maradufu, 1982). Furthermore, a water-soluble extract of a tropical shrub, *Margaritaria discoidea* was observed to induce high mortalities in nymphs and adults of *R. appendiculatus* (Kaaya et al., 1995).

However, derivatives of the Neem tree (*Azadirachta indica* A. Juss), which have been traditionally used by farmers in Asia and Africa to control insect pests of

household, agricultural and medical importance (Saxena, 1989; Schmutterer, 1990) appear to hold more promise for tick control. Kaaya et al. (2007) reported that Neem derivatives administered topically or systemically to host animals induced mortality, reduced fecundity and engorgement weights and deterred attachment of the tropical bont tick *Amblyomma variegatum* to host animals. The objective of this study was therefore to investigate the pathophysiological effects of Neem derivatives on the brown ear tick, *R. appendiculatus*, the vector for theileriosis (East coast fever), the most devastating cattle disease in eastern, central and southern Africa.

MATERIALS AND METHODS

The study was conducted at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. Rabbits were kept in small animal house and goats in animal pens but were released to graze outside daily for 2 to 3 h as explained elsewhere in this paper. The brown ear tick, *R. appendiculatus*, were reared on New Zealand white rabbits kept in metal cages as described by Dossa et al. (1996) and Solomon and Kaaya (1998). The rabbits were given rabbit pellets and water (*ad libitum*). Fresh Neem seeds; collected from ripe fruits harvested from Neem trees; were cleaned and dried in shade to 12 to 14% moisture content. Whole seeds were manually pounded using a large steel mortar-and-pestle to produce a fine Neem seed powder (NSP). Neem oil (NO) was obtained by cold-pressing whole seeds in a single screw vegetable oil expeller (IBG Monforts, GmbH & Co., Mönchengladbach, Germany). The concentration of Azadirachtin-A (Aza), the active compound in the Neem derivatives (NSP and NO) (Saxena, 1989; Schmutterer, 1990) were determined using high-pressure liquid chromatography and was found to be 5000 ppm in NSP and 850 ppm in NO. Peanut oil (PO) was used as a control. NO and PO were emulsified to various concentrations in water by using 1% Tween-80 as described by Kaaya et al. (2007).

Effects of Neem treatment on *R. appendiculatus* (*in vitro*)

Mortality induced by Neem oil (NO)

Larvae in glass vials which had been kept in an incubator maintained at 10°C were removed, counted, placed in nylon mesh tetra packs and immersed (in batches of 100) in 10% Neem oil or 10% Peanut oil for 5 min, then removed, dried on filter papers, placed in plastic petri dishes and incubated at 28°C and 85% relative humidity for 24 h to observe mortality.

Effects of NO on tick engorgement and reproduction

Three rabbits were exposed to 200 larvae and 50 adult female ticks per ear, after which 1 ml of 10% NO and similar concentrations of PO (control) were applied on the right rabbit ear (NO) and on the left ear (PO) using a fine brush. Ear bags were then applied as explained by Dossa et al. (1996) and Solomon and Kaaya (1998). Two days after exposure, the ear bags were opened and the ticks that had failed to attach were discarded. Thereafter, the ear bags were opened daily and ticks that had engorged and detached were carefully removed. Feeding periods, engorgement weights, moulting percentages and egg batch weights were recorded. To

determine feeding periods, the number of days taken by the ticks to engorge and detach from rabbit ears were recorded. To determine engorgement weights, larvae in groups of 50 and adults in groups of 10 were weighed after detachment. To determine moulting percentages (larvae), egg batch weights and egg hatchability, three groups of 50 larvae and 10 engorged adult females were placed on filter papers in Petri dishes and incubated at 28°C and 85% relative humidity (Rh). Larvae that hatched into nymphs and eggs that hatched into larvae were counted, whereas egg batch weights were determined by weighing eggs laid by the individual adult ticks.

Deterrence of tick attachment by NO

One millilitre of 10% NO and similar amounts and concentrations of PO were applied on the right rabbit ear (NO) and on the left ear (PO) using a fine brush, after which 200 larvae, 100 nymphs and 50 adult ticks were applied. Ear bags were then attached to the ears and ticks observed for 10 days after which the numbers of attached and unattached ticks were counted.

Effects of NO on hatchability of eggs

Five batches of 100 mg tick eggs were weighed and placed on filter papers (Whatman No. 1) in disposable petri dishes and wetted with 0.5 ml of undiluted NO or undiluted PO (control) and incubated at 28°C and 85% Rh for 30 days, after which the numbers of emerged larvae were counted.

Host-mediated effect of Neem on *R. appendiculatus*

Using the ear bag method (Solomon and Kaaya, 1998), sixteen 12 to 14 month-old male white 'Galla' goats, each weighing 30 to 40 kg, had their ears shaved and infested with 200 larvae, 100 nymphs, and 25 adult males + 25 adult females per goat. They were then kept individually in pens (1 m × 1 m) and provided with Lucerne and water daily (*ad libitum*) in the mornings and evenings, and released for grazing for 2 to 3 h daily. To investigate the effects of Neem on the ticks feeding on the goats, the goats were placed in 4 groups of 4 and treated as follows: Group 1- goats were fed on rabbit pellets mixed with 25% Neem seed powder (NSP); Group 2 goats were fed on rabbit pellets mixed with 50% NSP; Group 3 goats were fed pure (100%) NSP; Group 4 (control) were fed on 100% rabbit pellets (0% NSP). All concentrations were mixed with 5% molasses since Neem is very bitter and goats would hesitate to eat. The goats were allowed to eat *ad libitum*. Tick attachment, feeding periods, engorgement weights, moulting percentages, fecundity and egg hatchability were then determined as explained earlier. All animals were treated ethically and ethical clearance certificate was obtained from ICIPE before commencement of the experiments.

Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) and Ryan-Einot-Gabriel-Welsch (REGW) multiple range test to determine differences between experimental and control groups.

RESULTS

Application of 10% Neem oil on larvae of *R. appendiculatus* induced significant mortality as compared

to PO control. The 10% NO induced a mortality of 40% and PO control less than 1% (Figure 1a). Larval moulting was also significantly reduced by NO. Although the feeding periods were not reduced, the larval engorgement weights were significantly reduced (Figure 1b). The adult ticks which were smeared with 10% NO after attachment exhibited significant reductions in engorgement weights (388 mg control vs 295 mg NO) and significant reductions in egg weights (182 mg control vs 100 mg NO) (Figure 1b).

Neem oil (10%) smeared on rabbit ears deterred attachment by larvae and nymphs of *R. appendiculatus* compared to PO controls but had little effect on the adult ticks (Figure 2a). The attachment was reduced by 90% in larvae and 70% in nymphs. As shown in Figure 2b, feeding periods were longer in NO treated compared to PO control larvae. Eggs of *R. appendiculatus* which were exposed to 100% NO on filter papers and incubated at 28°C and 85% relative humidity for 30 days exhibited significant loss of viability as compared to controls exposed to PO in a similar manner (Figure 3).

Significant numbers of larvae and nymphs of *R. appendiculatus*, fed on goats maintained on a diet containing various concentrations of NSP were unable to attach and feed on the goats and many suffered significant mortalities. These effects increased with increasing concentrations of NSP in the goat diet (Figure 4). For instance, while 65% of control larvae attached, only 15% in the 50% NSP group and 11% in the 100% NSP group were able to attach (Figure 4). Nymphs were also affected but not as severely as the larvae. In the control group, 79% attached, while 36% in the 50% NSP group and 24% in the 100% NSP group attached. Nymphs suffered higher mortalities than larvae. Their control group had a mortality of about 17%, the 50% NSP group 35% and the 100% NSP group 45% (Figure 4).

Adult ticks fed on the goats maintained on a diet containing various concentrations of NSP had significantly reduced engorgement weights which reduced with increasing concentrations of NSP in the goat diet (Figure 5). In the adult ticks, the engorgement weights of the control group were 296 mg, that of 50% NSP group 234 mg and that of 100% NSP were 208 mg (Figure 5). Furthermore, the adult ticks produced significantly fewer eggs, as well as reduced egg batch weights and egg hatchability which increased with increasing concentrations of NSP in the goat diet (Figure 6).

DISCUSSION

In Australia, Rice (1993) reported that a monthly spray of ethanolic aza's (Gigi petsray ® 3,000 ppm aza) or weekly bathing in aqueous 1:20 Green Gold one ® controlled the bush tick *Ixodes holocyclus* and the cattle tick,

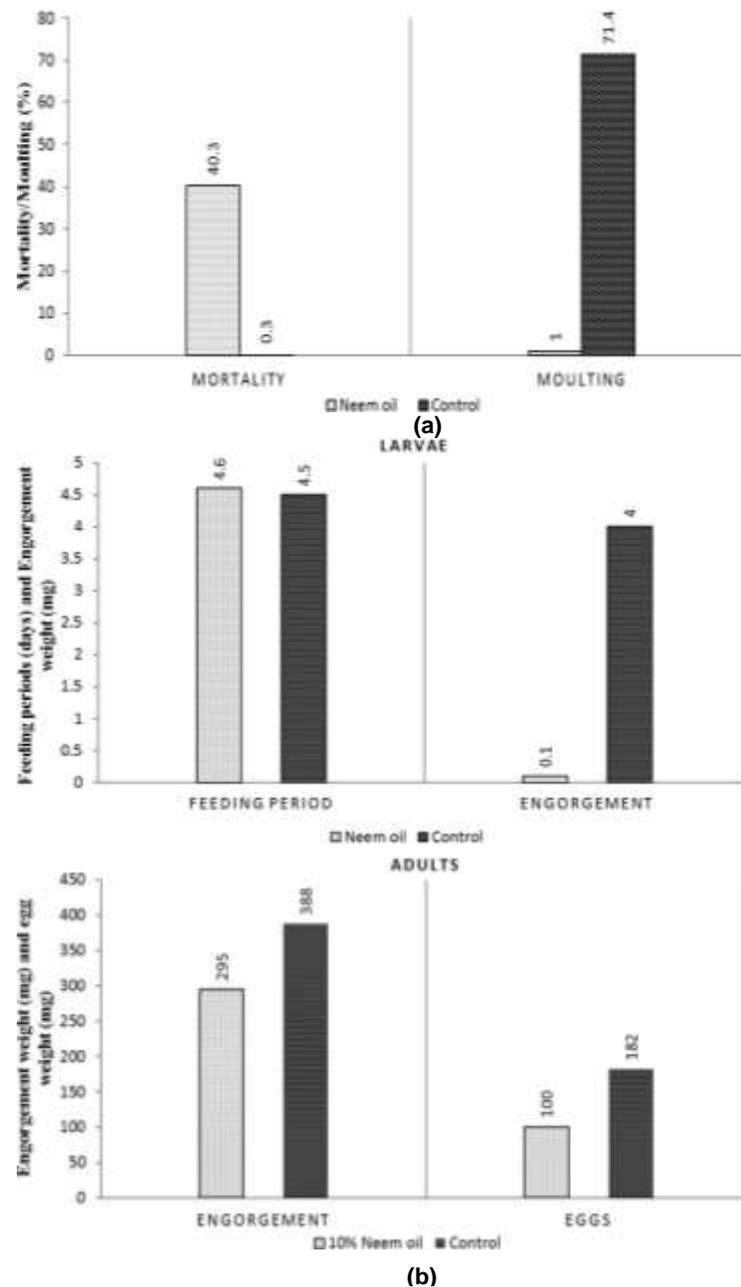
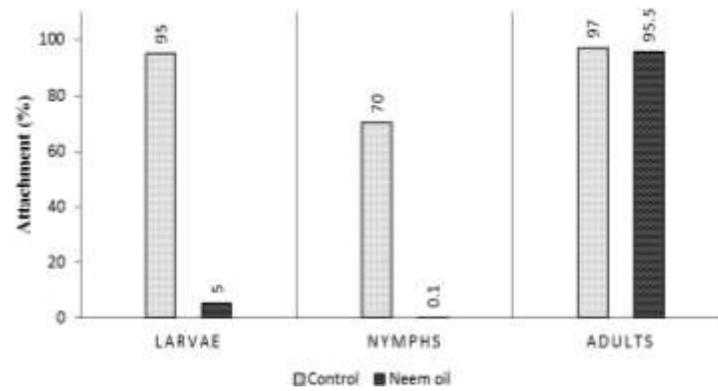


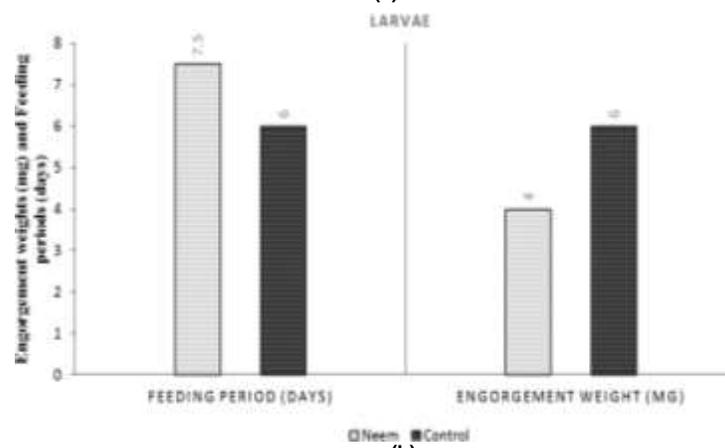
Figure 1. (a) Mortality and Moulting in *R. appendiculatus* larvae treated with 10% NO and control (PO) in the same concentration; (b) Engorgement weights (mg), feeding periods (days) in *R. appendiculatus* larvae and adult treated with 10% and control (PO).

Rhipicephalus (Boophilus) microplus, but was less effective against the brown dog tick, *Rhipicephalus sanguineus*. In this study, Neem derivatives when applied on tick eggs or on various developmental stages of the brown ear tick, *R. appendiculatus*, induced profound pathophysiological effects such as loss of egg viability, mortality, prolonged feeding periods, reduced

engorgement weights, difficulty in attachment, reduced moulting, reduced fecundity and egg hatchability. The overall effect likely reduces tick survival and populations on cattle and in the environment. Furthermore, ticks maintained on goats fed on a diet containing Neem seed extract exhibited high mortality, reduced engorgement weights and egg hatchability. Landau et al. (2009)



(a)



(b)

Figure 2. (a) Deterrence of attachment of different instars of ticks on rabbit ears by 10% NO. (b) Feeding periods and engorgement weights in larvae of *R. appendiculatus* fed on rabbit ears smeared with 10% NO and PO controls.

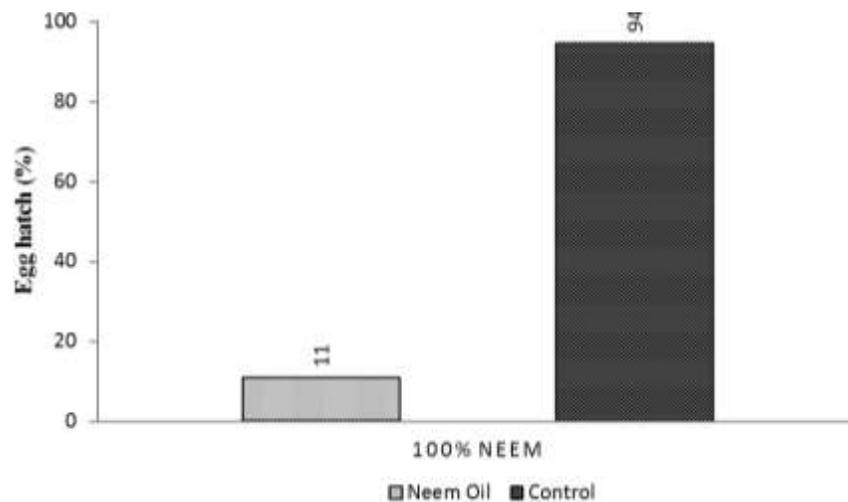


Figure 3. Hatchability of eggs of *R. appendiculatus* exposed to NO and to control (PO). The oils were applied directly on filter papers on which 100 mg of eggs were incubated.

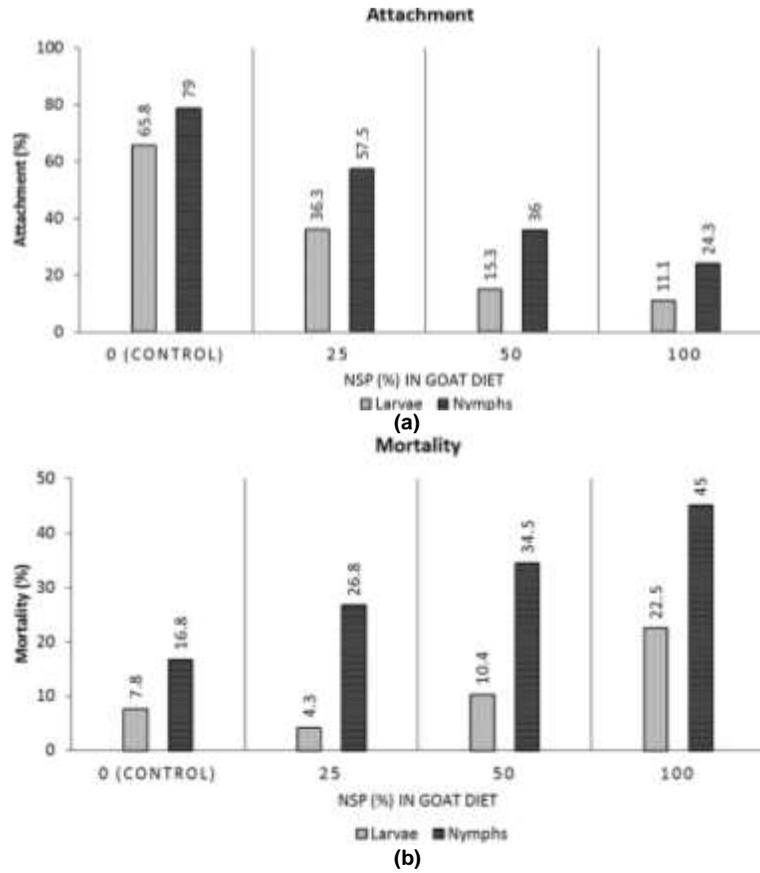


Figure 4. (a) Attachment (%) and (b) mortality (%) of larvae and nymphs of the brown ear tick, *R. appendiculatus* fed on goats maintained on a diet containing NSP.

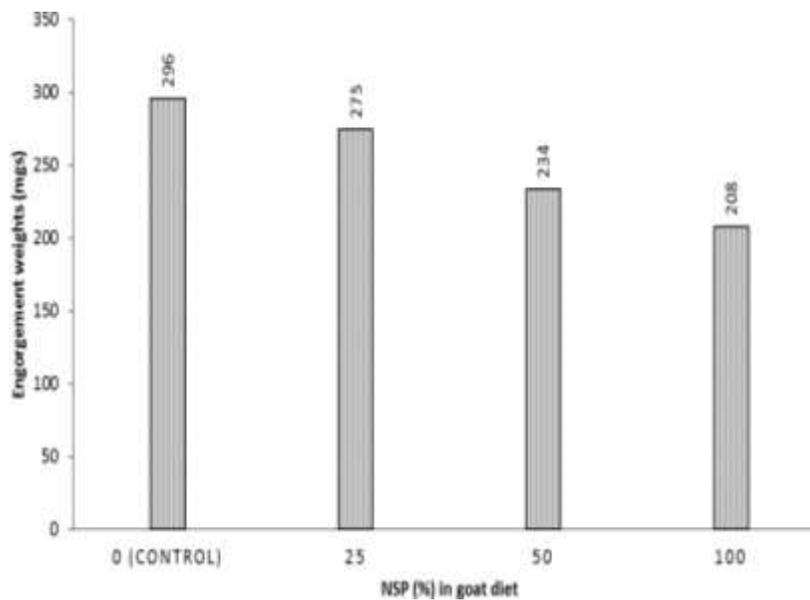


Figure 5. Engorgement weights (mg) of adults of *R. appendiculatus* fed on goats maintained on a diet containing various concentrations of NSP.

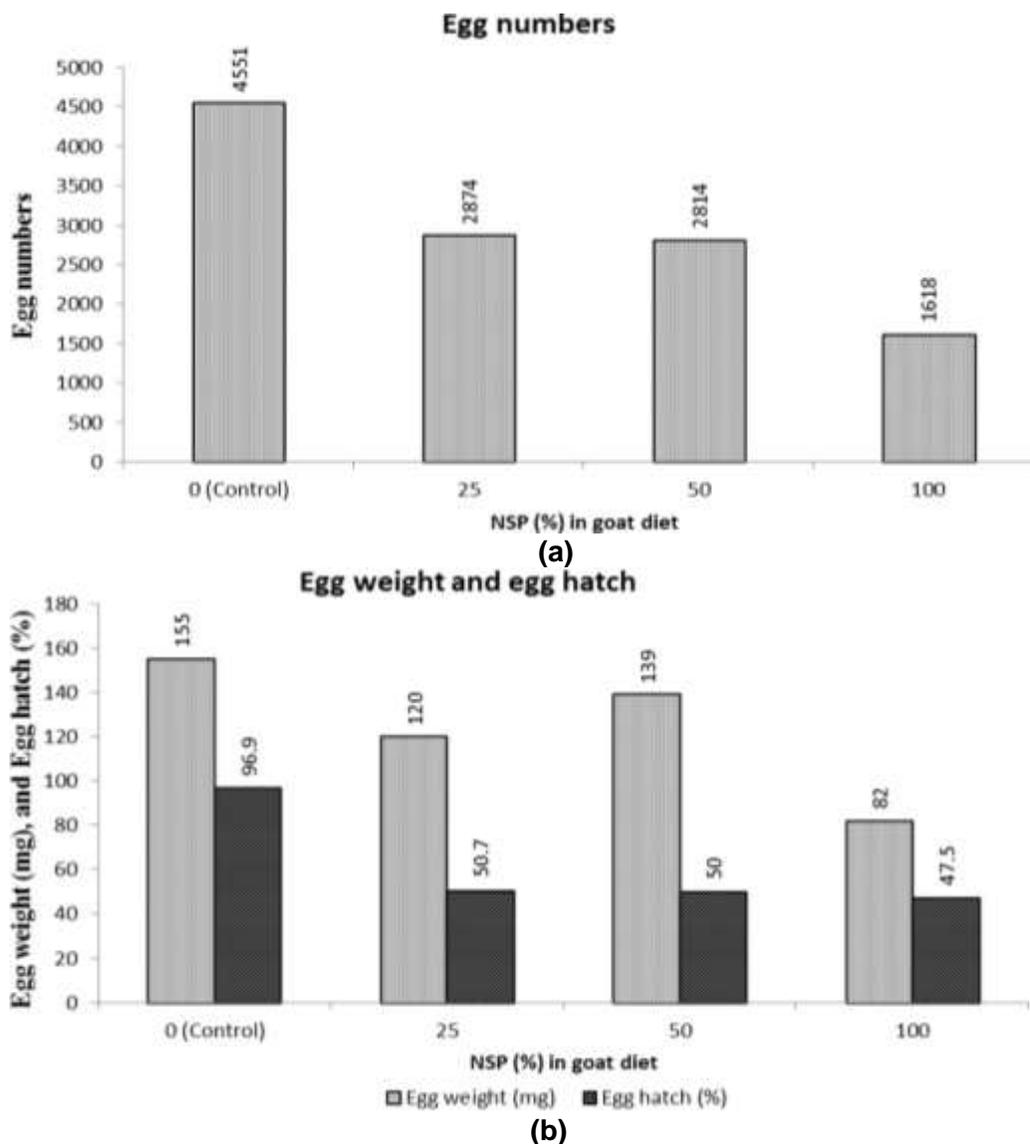


Figure 6. Egg numbers, egg weights (mg) and egg hatch (%) in ticks maintained on goats fed on a diet containing different concentrations of NSP.

reported that Neem extract mixed with sheep diet significantly reduced engorgement weights of the American dog tick *Dermacentor variabilis* fed on the sheep.

A 25% Neem oil applied on de-ticked Zebu cattle grazing in tick-infested pastures significantly reduced the number of immature and adult *A. variegatum*, *R. appendiculatus* and *Rhipicephalus (Boophilus) decoloratus* ticks attaching on cattle for a period of 4 to 5 days (Kaaya et al., 2007). Likewise, Schwalback et al. (2003) reported that Neem (*Azadirachta indica*) seed extract significantly reduced tick populations on goats when sprayed as 10% aqueous extract. Furthermore,

Webb and David (2002) sprayed 5% Neem seed water extract on de-ticked cattle which were grazed alongside water sprayed control cattle and observed a significant reduction in on-host tick populations on the Neem sprayed cattle and concluded that Neem seed extract is effective in controlling ectoparasites on livestock.

Similar pathophysiological effects have been reported in other arthropod pests. For instance, Neem derivatives have been reported to cause physiological effects, such as repellence, feeding and oviposition deterrence, growth inhibition, mating disruptions, reduced fecundity and egg hatchability in a number of insect pests (Saxena, 1989; Schumtterer, 1990). The deterrence of tick attachment

and the antifeedant effect of Neem derivatives which resulted in significantly reduced engorgement weights in all tick instars is likely to reduce blood loss in cattle. Figure 5 shows that more nymphs than larvae were able to attach and the lower compartment shows that more nymphs died probably because they ingested more blood than larvae and hence more Neem derivatives. Thus, even ticks that managed to attach are harmed by the Neem derivatives contained in the host animal blood.

In this study, Neem oil applied on rabbit ears and Neem seed powder fed on goats significantly reduced tick attachment and feeding by prolonging feeding periods and reduced tick engorgement weights. Kaaya et al. (2007) reported similar observations in the tick *A. variegatum* exposed to Neem derivatives by direct application or by feeding the ticks on goats maintained on the Neem-containing diet. In the current investigation, Neem derivatives have shown great potential as effective tick control agents. Neem trees are grown in many African countries and their derivatives in various forms are used for control of various insect pests (Saxena, 1989). Furthermore, Neem products are environmentally safe and have been reported to have negligible side effects on non-target organisms (Jacobson, 1989; Schmutterer, 1995). The reduction in engorgement weights will reduce blood loss common in heavy tick infestations. Furthermore, since *Theileria* parasites are transmitted transstadially (That is, infection picked by larva is transmitted by nymph and that picked by nymph is transmitted by an adult) (Norval et al., 1992), reduction of tick moulting is likely to affect the transmission of *Theileria* parasites. The low cost of Neem derivatives and the environmental safety renders the use of Neem highly attractive for tick management by resource-limited African livestock farmers. A review by Borges et al. (2011) indicated that plant extracts including Neem offer promising alternatives to chemicals.

In the early 2000s, the Kenya Government approved ECF immunization of cattle in all, except marginal areas. This method involves infection of cattle with East Coast Fever parasites followed by treatment (Homewood et al., 2006). Economic analyses have demonstrated that ECF immunization, when incorporated in the integrated ECF control makes an important contribution to reducing the overall cost of control (Gachohi et al., 2012). Immunization of cattle against ECF enables reduction of the use of chemical acaricides for the control of ticks and ECF. Neem derivatives may be used to replace or limit the application of chemical acaricides thus saving farmers the cost of combating ticks and ECF control. It is also likely to protect the environment from chemical pollution.

Abbreviations: AZA, Azadirachtin-A; DDT, dichlorodiphenyltrichloroethane; ECF, East Coast Fever; NO, Neem oil; NSP, Neem seed powder; PO, peanut oil.

Ethical treatment of animals

The authors declare that all animals used in the experiments were treated ethically according to international regulations that are followed at ICIPE, Kenya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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