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Full Length Research Paper

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

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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

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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 Mc Leod, 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 *Ambyomma 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 holocylus* and the cattle tick,

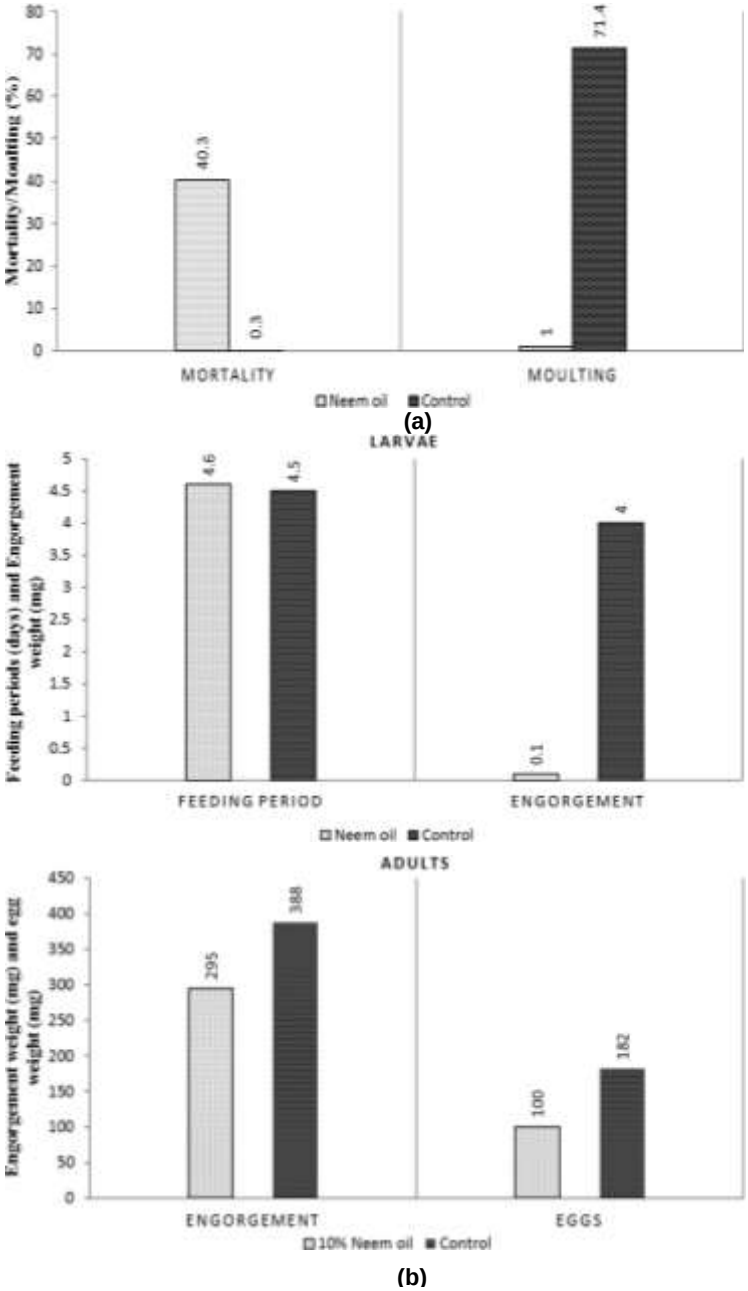
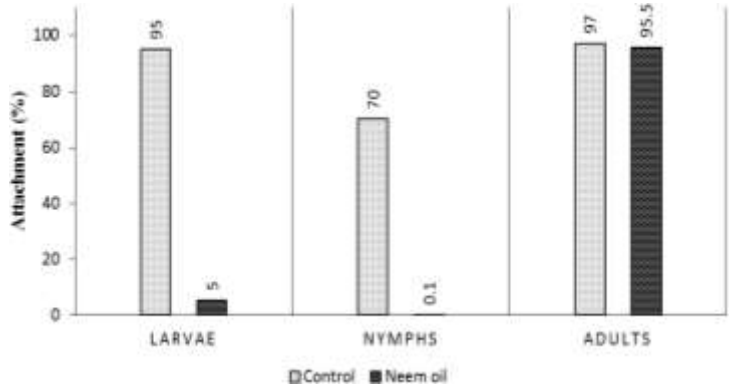
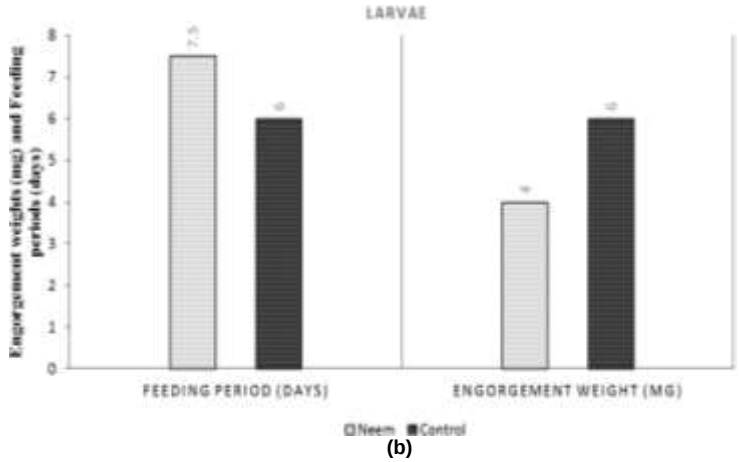


Figure 1. (a) Mortality and Moulting in *R. appendiculatus* larvae treated with 10% NO and control (PO) in the same concentration; **(b)** Engorgement weights (mg), feeding periods (days) in *R. appendiculatus* larvae and adult treated with 10% and control (PO).

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.

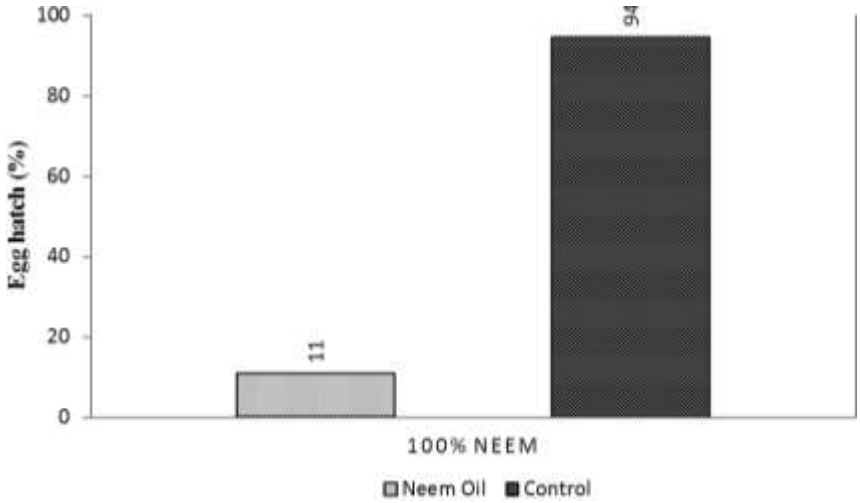


Figure 3. Hatchability of eggs of *R. appendiculatus* exposed to NO and to control (PO). The oils were applied directly on filter papers on which 100 mg of eggs were incubated.

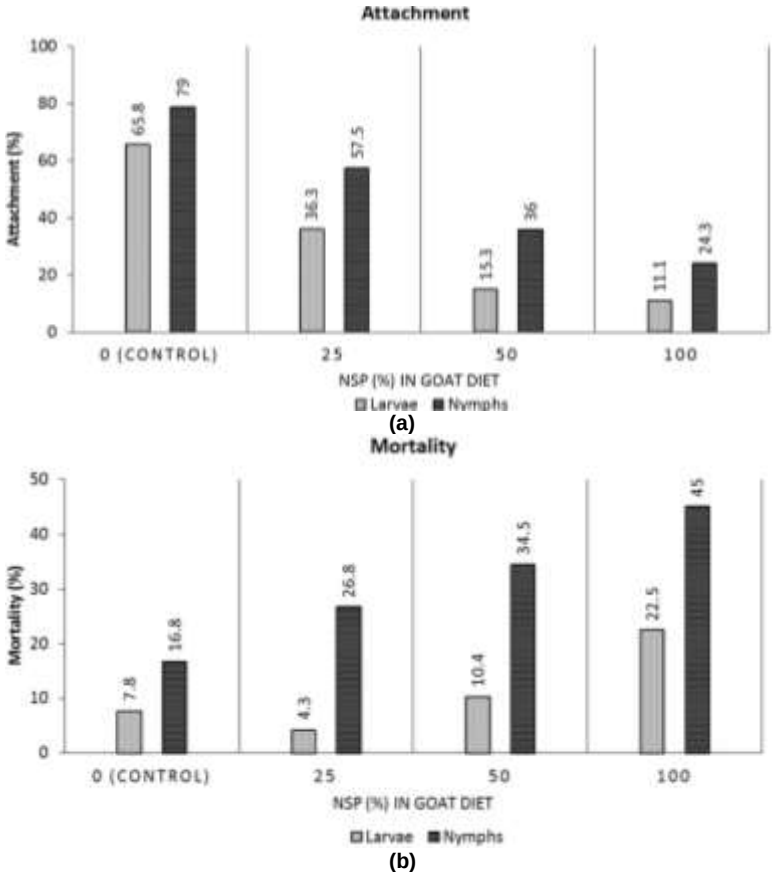


Figure 4. (a) Attachment (%) and (b) mortality (%) of larvae and nymphs of the brown ear tick**,** *R. appendiculatus* fed on goats maintained on a diet containing NSP.

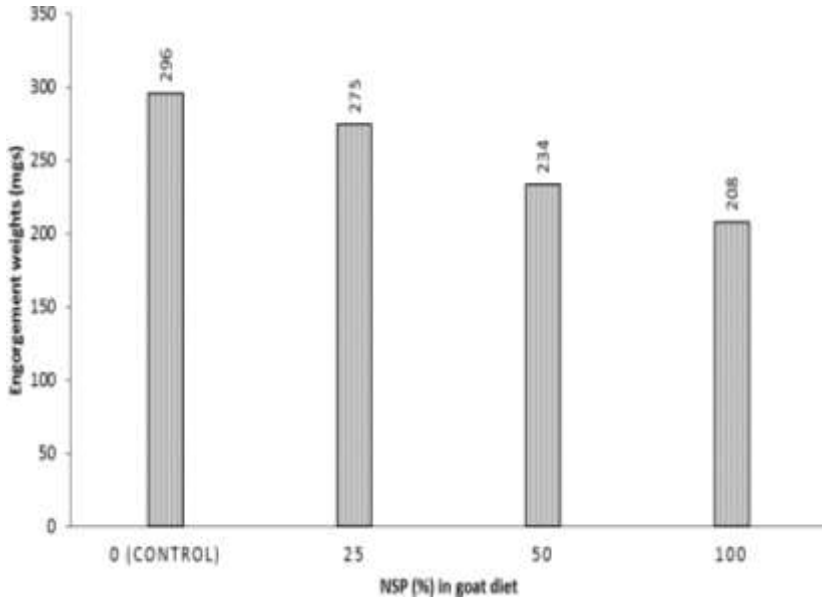


Figure 5. Engorgement weights (mg) of adults of *R. appendiculatus* fed on goats maintained on a diet containing various concentrations of NSP.

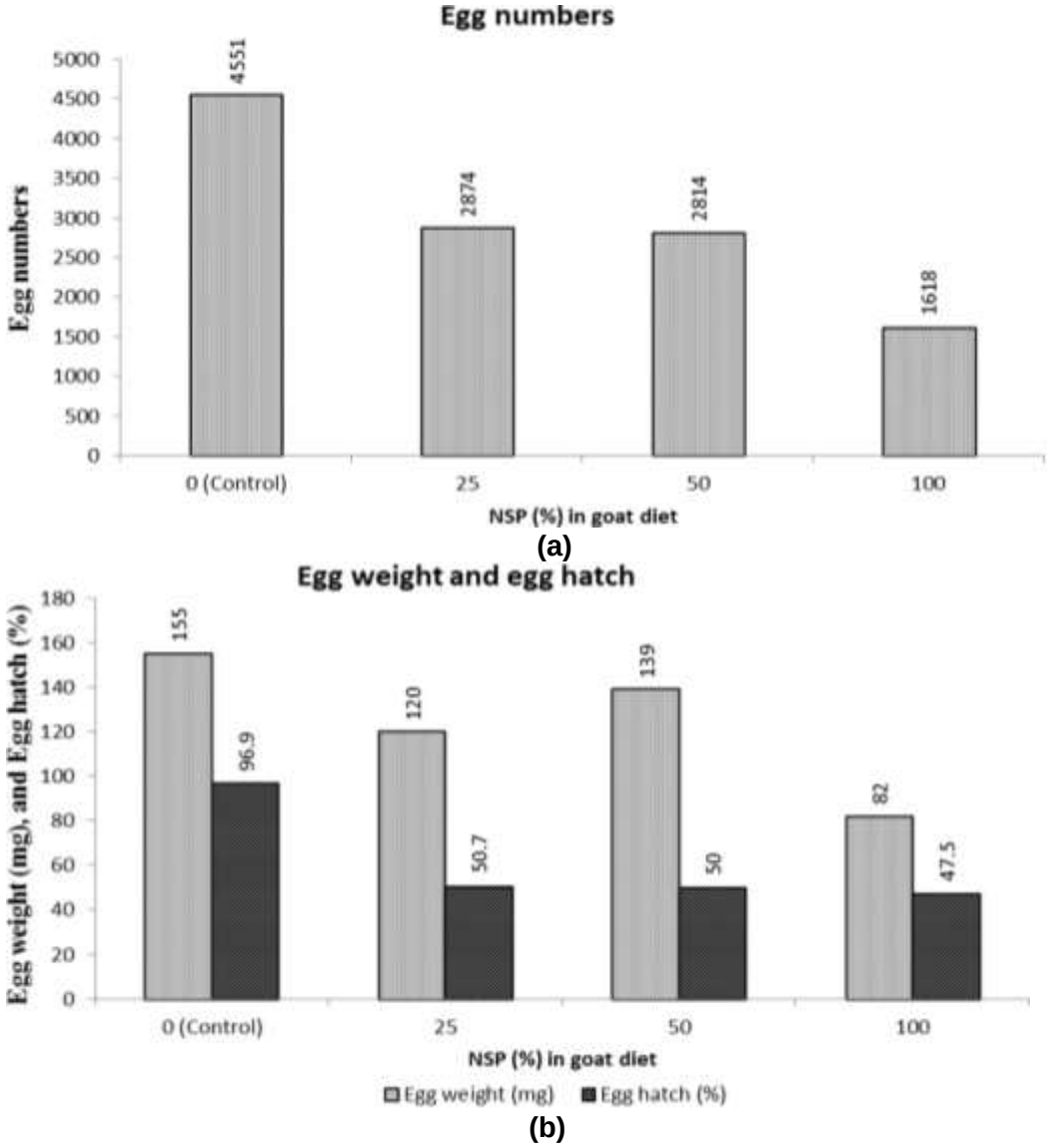


Figure 6. Egg numbers, egg weights (mg) and egg hatch (%) in ticks maintained on goats fed on a diet containing different concentrations of NSP.

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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Journal of Entomology and Nematology

Full Length Research Paper

Acaricidal activity of *Chrozophora oblongifolia* on the two spotted spider mite, *Tetranychus urticae* Koch

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The acaricidal activity of *Chrozophora oblongifolia* (Delile) Spreng. (Euphorbiaceae) extracts collected from Dakhla Oasis, Western Desert of Egypt was examined against larvae and adult females of *Tetranychus urticae* Koch (Acari: Tetranychidae). Acaricidal activity-guided isolation of methylene chloride, ethyl acetate, butanol fractions resulted in separation and identification of *p*-hydroxybenzaldehyde (1), 3,5-dimethoxy-4-hydroxybenzaldehyde (2), scopoletin (3), amentoflavone (4), apigenin 7-O-β-D- glucopyranoside (5) and apigenin 7-O-β-D-[2",6"-bis(4-hydroxy-E-cinnamoyl)] glucopyranoside (6). The isolated compounds were identified by MS and NMR spectral analyses. The susceptibility of the larvae and adult females of *T. urticae* Koch to the tested isolated compounds revealed that apigenin 7-O-β-D-[2",6"- bis(4-hydroxy-E-cinnamoyl)] glucopyranoside (6) and apigenin 7-O-ß-D- glucopyranoside (5) isolated from butanol fraction (most effective fraction) exhibited a high degree of acaricidal activity using leaf-dipping technique against larvae after 7- days of exposure, respectively.

Key words: Acaricidal activity, phytochemistry, *Tetranychus urticae* Koch, *Chrozophora oblongifolia*, Euphorbiaceae.

INTRODUCTION

Two-spotted spider mite (*Tetranychus urticae* Koch) is a phytophagous pest that causes significant yield losses in many agricultural crops in Egypt, including cotton, vegetables, fruits and ornamentals (Dawidar et al., 2014). *T. urticae* feeds by puncturing cells and draining the contents, producing a characteristic yellow-brown speckling on the leaf surface. They also produce silk webbing which is clearly visible at high infestation levels (Salman et al., 2014). The overuse of synthetic acaricides can lead to serious adverse effects against humans and

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the environment, as well as non-target organisms including beneficial insects and mites that prey on pests (Geng et al., 2014). Because of this problem, green pesticides of botanical sources were considered to be a safer alternative approach to the widest spread synthetic one and have great demand by several scientists (Koul et al., 2008).

Chrozophora oblongifolia (Delile) Spreng. (Euphorbiaceae) was recorded in Western Desert (Alshamy, 2016), Eastern Desert (Salama et al., 2014; Shaltout et al., 2010), Gabel Elba (Abd El-Ghani and Abdel-Khalik, 2006) and South Sinai (Abdel Ghani and Amer, 2003). It is a perennial, erect shrub, subshrub or woody herb up to 1 m high with inflorescences up to 1.5 cm long and described as a much-branched under a shrub, with stems stout and woody below, but sometimes herbaceous and dying after flowering in the first year; stems rather harshly white or tawny stellate-pubescent. Leaves are distinctly petioled, ovate-rhomboid or oblong to lanceolate (Ahmed et al., 2014). *C. oblongifolia* extracts exhibited strong antihepatotoxic (Abdel-Sattar et al., 2014), antioxidant antiviral and antimicrobial activities (Kamel et al., 2016). This phytochemical investigation of *C. oblongifolia* led to isolation and identification of phytochemicals 1-octacosanol, lupeol, *p*-hydroxybenzoic acid, methyl gallate and amentoflavone (Kamel et al., 2016).

The objective of this study was to investigate the phytochemistry and the acaricidal activity of *C. oblongifolia* extracts and secondary metabolites.

MATERIALS AND METHODS

Instruments

NMR spectra were recorded on a Bruker AMX 400 and 500 instrument standard pulse sequences operating at 400 and 500 MHz in ^{1}H-NMR and ^{13}C-NMR were recorded at 125 MHz. Chemical shifts are given in δ (ppm) relative to TMS as internal standard material and the coupling constants (*J*) are in Hz. HSQC, HMBC (H-H) COSY and NOESY were recorded at 500 MHz. GC/MS analysis was performed on a Varian GC interfaced to Finnegan SSQ 7000 Mass selective Detector (SMD) with ICIS V2.0 data system for MS identification of the GC components.

Chemicals

Columns chromatography (CC) was performed using silica gel F254 (230-400 mesh) or polyamide 6. Thin layer chromatography and preparative TLC were performed on silica gel (Kieselgel 60, F 254) of 0.25 mm thickness. Solvents of hexane, methylene chloride, ethyl acetate, butanol and methanol were obtained from Adwic Company.

Plant material

C. oblongifolia (Delile) Spreng was collected in Dakhla: Tennida veillage (Lattitude 25° 30.518 N and Longitude 029° 19.964 E) on April, 2016 and identified by the second author according to Boulos (2000) and Boulos (2009).

Extraction and isolation

The air dried powdered whole plant (1 kg) was macerated in a mixture of organic solvents of methylene chloride/methanol (1:1), the filtrate was evaporated to its 1/3 volume and then diluted by water and exhaustively extracted by hexane, then methylene chloride, ethyl acetate and finally by butanol. All the extracts were collected, dried over anhydrous sodium sulphate and evaporated to give hexane fraction (13.71 g), methylene chloride fraction (2.41 g), ethyl acetate fraction (3.74 g) and butanol fraction (4.42 g).

The methylene chloride fraction (2.41 g) was chromatographed over silica gel column chromatography using mixtures of hexane/ethyl acetate and methylene chloride/methanol of increasing polarities. The effluents were combined into ten sub-fractions based on their TLC pattern. Sub-fraction III was further purified on silica gel preparative TLC developed by a mixture of hexane/toluene/ethyl acetate (65:25 v/v) to afford a pure compound (1) (190 mg, R_f = 0.16). Sub-fraction V was purified on silica gel preparative TLC using a mixture of toluene/ethyl acetate (84:16 v/v) as a mobile phase and give compound (2) (160 mg, R_f = 0.13). Sub-fraction VIII have been separated on PTLC silica gel plates using a mixture of toluene/ethyl acetate (77:23 v/v) to yield compound (3) (80 mg, R_f = 0.32).

Ethyl acetate fraction (3.74 g) was fractionated by chromatography on polyamide 6 CC. The column was eluted with water, water–methanol (1:1), methanol, methanol-acetone (1:1), acetone, acetone-ammonium hydroxide (1:1) and ammonium hydroxide. The obtained fractions (500 ml of each fraction) gave ten sub-fractions according to their TLC pattern step gradient. Sub-fraction VIII was chromatographed on PTLC silica gel plates using ethyl acetate-methanol-water (8.5:1.2:0.3) as a developing system to afford compound (4) (225 mg, R_f = 0.52).

Butanol fraction (4.35 g) was separated by polyamide 6 CC using the same previous method to give ten subfractions. Separation of subfraction III was applied on PTLC Silica gel plates using EtOAc–methanol–H_2O (20:4:1) as eluting system to afford compound (5), (201mg, R_f = 0.31). Subfraction VIII was purified on silica gel PTLC developed by EtOAc–methanol–H_2O (42:7:1) to give compound (6), (105 mg, R_f = 0.36).(Figure 1)

p-Hydroxy benzaldehyde (1)

Yellow powder, ^{1}H-NMR ($CDCl_3$): δ_H 7.81 (1H, d, J = 8.5 Hz, H-2/H-6); 6.95 (1H, d, J = 8.5 Hz, H-3/H-5); 9.87 (1H, s, CHO).

3,5-Dimethoxy-4-hydroxybenzaldehyde (2)

Yellow solid, ^{1}H-NMR ($CDCl_3$): δ_H 9.81 (1H, s, CHO); 7.15 (2H, s, H-2/H-6); 6.10 (1H, br.s); 3.97 (6H, s, 2 OCH_3); EI-MS; *m/z* (rel. int.) 182 (100%) $[M^+]$, 181 (4.2%) $[M\text{-}H]^+$, 167 (14%) $[M\text{-}CH_3]^{+\cdot}$, 151 (3%) $[M\text{-}OCH_3]^{+\cdot}$, 139 (7%) $[C_7H_7O_3]^{+\cdot}$, 111 (16%) $[C_7H_6O_2]^+$.

Scopoletin (3)

Colourless oily material, ^{1}H-NMR ($CDCl_3$): δ_H 7.60 (1H, d, J = 9.5 Hz, H-4); 6.92 (1H, s, H-5); 6.85 (1H, s, H-8); 6.27 (1H, d, J = 9.5 Hz, H-3); 3.96 (3H, s, OCH_3).

***Amentoflavone* (4)**

Amorphous yellow power, ^{1}H-NMR (CD_3OD): Unity I: δ_H 6.61 (s, 1H, H-3); 6.26 (s, 1H, H-6); 7.65 (d, 2H, J = 8.8 Hz, H-2'/H-6'); 6.61 (d, 2H, J = 8.8 Hz, H-3'/H-5'); Unity II: δ_H 6.61 (s, 1H, H-3); 6.14 (d, 1H, J = 2.1 Hz, H-6); 6.15 (d, 1H, J = 2.1 Hz, H-8); 8.23 (d, 1H, J = 2.3 Hz, H-2'); 7.09 (d, 1H, J = 8.7 Hz, H-5'); 7.89 (dd, 1H, J = 8.7 and 2.3 Hz, H-6'). ^{13}C-NMR (CD_3OD): Unity I: δ_C 166.5 (C-2); 103.1 (C-3); 183.7 (C-4); 163.0 (C-5); 103.2 (C-6); 165.5 (C-7); 104.9 (C-8); 156.6 (C-9); 108.3 (C-10); 121.6 (C-1'); 129.3 (C-2'/C-6'); 116.8 (C-3'/C-5'); 162.5 (C-4'); Unity II: δ_C 166.5 (C-2); 103.5 (C-3); 183.9 (C-4); 163.6 (C-5); 100.5 (C-6); 163.6 (C-7); 95.4 (C-8); 159.4 (C-9); 108.3 (C-10); 123.2 (C-1'); 132.8 (C-2'); 124.5 (C-3'); 162.2 (C-4'); 120.4 (C-5'); 127.8 (C-6').

***Apigenin 7-O-ß-D- glucopyranoside* (cosmosiin) (5)**

Amorphous yellow power, ^{1}H-NMR (DMSO-d6): δ_H 7.86 (2H, d, J = 8.4 Hz, H-2' /H-6'); 6.89 (2H, d, J = 8.4 Hz, H-3' /H-5'); 6.62 (1H, s, H-3), 6.81 (1H, d, J = 2 Hz, H-8); 6.49 (1H, d, J = 2 Hz, H-6); 5.09 (1H, d, J = 6.8 Hz, H-1"); 3.96 (1H, dd, J = 11.6, 1.2 Hz, H-6″b); 3.70 (1H, dd, J = 11.6, 5.6 Hz, H-6″a); 3.60-3.37 (4H, m, H-2″,3″,4″,5″).

***Apigenin 7-O-β-D-[2",6"- bis(4-hydroxy-E-cinnamoyl)]glucopyranoside* (anisofolin-B) (6)**

Yellow powder; ^{1}H-NMR (CD_3OD): δ_H 6.6 (1H, s, H-3); 6.47 (1H, d, J = 1.8 Hz, H-6); 6.78 (1H, d, J = 1.8 Hz, H-8); 7.84 (2H, d, J = 8.4 Hz, H-2′ /H-6′); 6.91 (2H, d, J = 8.4 Hz, H-3′ /H-5′); 7.44 (2H, d, J = 8.6 Hz, H-2‴ /H-6‴); 6.79 (2H, d, J = 8.6 Hz, H-3‴ /H-5‴); 7.60 (1H, d, J = 16 Hz, H-7⁗); 6.31 (1H, d, J = 16 Hz, H-8⁗); 7.17 (2H, d, J = 8.5 Hz, H-2⁗ /H-6⁗); 6.61 (2H, d, J = 8.5 Hz, H-3⁗ /H-5⁗); 7.50 (1H, d, J = 15.9 Hz, H-7⁗); 6.26 (1H, d, J = 15.9 Hz, H-8⁗); 5.07 (1H, d, J = 7.1 Hz, H-1″); 3.54 (1H, m, H-2″); 3.50 (1H, m, H-3″); 3.40 (1H, m, H-4″); 3.65 (1H, m, H-5″); 3.72 (1H, m, H-6″a); 4.25 (1H, m, H-6″b); ^{13}C-NMR (CD_3OD): δ_C 164.7 (C-2); 104.0 (C-3); 184.0 (C-4); 160.1 (C-5); 101.2 (C-6); 163.2 (C-7); 96.1 (C-8); 156.4 (C-9); 107.0 (C-10); 122.8 (C-1'); 129.6 (C-2'/C-6'); 117.1 (C-3'/C-5'); 160.4 (C-4'); 127.1 (C-1‴); 131.1 (C-2‴/C-6‴); 116.8 (C-3‴/C-5‴); 158.9 (C-4‴); 146.3 (C-7‴); 114.8 (C-8‴); 169.0 (C-9‴); 126.9 (C-1⁗); 131.0 (C-2⁗/C-6⁗); 116.7 (C-3⁗/C-5⁗); 158.7 (C-4⁗); 147.0 (C-7⁗); 114.4 (C-8⁗); 166.7 (C-9⁗); 101.6 (C-1″); 74.1 (C-2″); 74.7 (C-3″); 71.3 (C-4″); 77.8 (C-5″); 62.4 (C-6″).

Maintenance of spider mite colony

Colony of spider mite, *T. urticae* Koch was reared under laboratory condition (25±2°C and 60±5% R.H) at plant protection research institute branch, Dakahlia Governorate. This colony was isolated from heavily infested castor oil plant leaves and reared on fresh one. These leaves were cleaned and placed on moisten cotton wool pad in Petri dishes. This colony was left for one year under the precious conditions in order to get a homogenous and sensitive colony. Spider mites individual were transferred to the leaves by the aid of fine camels hair brush. Breeding leaves were changed twice weekly at the summer and once weekly at the winter. Adding water was done twice daily to prevent escaping of *T. urticae* individuals.

Assessment of acaricidal activity

In this respect, laboratory experiments were conducted to evaluate the activity of tested plant extracts and its isolated compounds against *T. urticae* mobile stages (larvae and adult females). The leaf-dip technique was used (Dittrich, 1962).

The indication of mortality was chosen as the failure of mites to respond positively by leg movement followed light brooding with a fine brush. Mortality percentages were determined and corrected by using Abotts (1925) formula and they are statistically analyzed to estimate LC_{50}, LC_{90} and slope values according to Finney (1971). Toxicity index was computed for different extracts and their isolated compounds by comparing these materials with the most effective extracts or isolated compounds using Sun's (1950) equation.

$$\text{Toxicity index} = \frac{LC_{50} \text{ of compound A}}{LC_{50} \text{ of compound B}} \times 100$$

Where, A is the most effective compound; B is the tested compound.

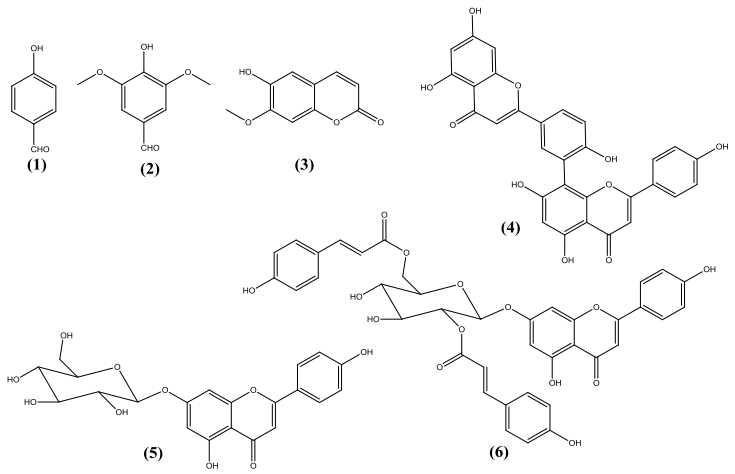

Figure 1. structures of the isolated compounds.

RESULTS AND DISCUSSION

The whole plant parts of *C. oblongifolia* were processed to give four different fractions of different polarities: hexane, methylene chloride, ethyl acetate and butanol fractions. These fractions were screened for their acaricidal activity against the larvae and adult females (mobile stages) of *T. urticae* after 7-days of exposure to find out the most effective fractions and search for its bioactive ingredients. Table 1 revealed that the most effective fractions were butanol, ethyl acetate and methylene chloride against both larvae and adult females of *T. urticae* after 7-days of treatment, respectively.

Chromatographic separation using column and thin layer chromatography of these fractions resulted in isolation of six compounds, which were characterized by MS and NMR spectroscopy. Three shikimates were isolated from methylene chloride fraction and identified as *p*-hydroxy benzaldehyde (1), 3,5-dimethoxy-4-hydroxybenzaldehyde (2) and scopoletin (3). Compound (1) gave the characteristic ^{1}H-NMR spectrum possessing the same substitution pattern of AA'BB' system of *p*-hydroxy benzaldehyde, which was confirmed by comparing its spectra with those reported by Riaz et al. (2013). EI-MS spectrum of compound (2) showed a molecular ion peak at m/z 182 corresponding to [$C_9H_{10}O_4$]. The fragmentation pattern as well as ^{1}H-NMR spectral data suggested that compound (2) is 3,5-dimethoxy-4-hydroxybenzaldehyde which was compared with the previously published by Tripathi et al. (2010) and found to be the same. The ^{1}H-NMR spectrum of compound (3) showed two doublets with coupling constant 9.5 Hz at δ 6.27 and 7.60 ppm characteristic of coumarins, in addition to two aromatic singlets protons at δ 6.92 and δ 6.85 ppm and methoxyl group singlet at δ 3.96 ppm which was characteristic to scopoletin. Coumarin (3) was assigned by comparison its ^{1}H-NMR data with those reported by Dawidar et al. (2009).

Examination of ^{1}H and ^{13}C-NMR spectra of compound (4) which was isolated from ethyl acetate fraction revealed that it belongs to a biflavonoid of apigenin moiety which identified as amentoflavone. This biflavonoid was isolated and characterized previously from the same plant species by Kamel et al. (2016).

Compounds (5) and (6), isolated from butanol fraction were found to be belong to apigenin 7-O-glycosides, as established by ^{1}H and ^{13}C-NMR spectra, with the sugar anomeric proton signals at δ 5.09 (1H, d, J=6.8 Hz) and δ 5.08 ppm (1H, d, J=7.1 Hz), respectively which were assigned to glucopyranosyl moiety for both, indicating that compound (5) is apigenin 7-O-β-D- glucopyranoside (cosmosiin). Additionally, the spectrum of 6 contained signals of two *p*-coumaroyl moieties. So, structure of compound (6) was characterized as apigenin 7-O-β-D-[2",6"- bis(4-hydroxy-E-cinnamoyl)]glucopyranoside by H-H COSY, NOESY, HSQC and HMBC. Compounds (5) and (6) were previously isolated from *Chrozophora plicata* by Riaz et al. (2014).

It is worthwhile mentioning here, that this is the first report of *p*-hydroxybenzaldehyde (1), 3,5-dimethoxy-4-hydroxybenzaldehyde (2), scopoletin (3), cosmosiin (5) and apigenin 7-O-β-D-[2",6"- bis(4-hydroxy-E-cinnamoyl)] glucopyranoside (6) from *C. oblongifolia*.

Acaricidal effect of *C. oblongifolia* fractions and isolated compounds to larvae and adult females of *Tetranychus urticae* (Koch) after 7-days of exposure

Results in Table 1 showed the toxic action of plant extracts to larvae and adult females of *T. urticae* after 7-days of exposure. The butanol fraction was the most effective at the LC_{50} and LC_{90} levels, followed by ethyl acetate fraction, methylene chloride fraction and hexane fraction for both larvae and adult females using leaf-dipping technique. Comparing the slopes values, butanol fraction flattest toxicity line and methylene chloride fraction had the steepest one in case of larvae and fluctuated by increasing from hexane fraction to ethyl acetate fraction in case of adult females. The other fractions lines came between these two fractions (Table 1).

On the basis of the toxicity index it was observed that the butanol fraction was the most effective fraction against larvae and adult females of *T. urticae* after 7-days of treatment followed by ethyl acetate fraction, methylene chloride fraction and hexane fraction was the least toxic fraction.

The acaricidal efficiency of any plant extract depends up on the chemical constituents of every extract contains. The trial to separate and isolate the major effective metabolites and searching for promising acaricidal compounds is our main goal. Table 2 showed the susceptibility of the larvae and adult females of *T. urticae* to the tested isolated compounds. Taking the toxicity index in consideration, data revealed that anisofolin-B (6) which belong to flavonoids glycosides isolated from butanol fraction (most effective fraction) exhibited a high degree of efficiency against larvae after 7-days of exposure, followed by cosmosiin (5), amentoflavone (4), *p*-hydroxy benzaldehyde (1), 3,5-dimethoxy-4-hydroxybenzaldehyde (2) and scopoletin (3). The LC_{50} values were 109.68, 120.40, 209.90, 1016.04, 1536.96 and 3834.16 ppm, respectively. However, cosmosiin (5) which belong to flavonoids glycosides isolated from butanol fraction (most effective fraction) was the most effective at the LC_{50} level against adult females of *T. urticae*, followed by anisofolin-B (6), amentoflavone (4), *p*-hydroxy benzaldehyde (1), 3,5-dimethoxy-4-hydroxybenzaldehyde (2) and scopoletin (3). The LC_{50} values of these tested isolated compounds were: 237.68, 278.59, 380.03, 1101.26, 1738.95 and 7943.96 ppm,

Table 1. Toxicity of plant fractions against larvae and adult females of *T. urticae* after 7 days of treatment.

Plant extract	Larvae				Adult females			
	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value
Hexane fraction	1639.77 1070.72 3793.93	10413.89 6268.28 82671.00	1.596±0.339	12.6	2120.72 1304.34 8030.00	12978.32 4564.91 370964.21	1.629±0.446	14.7
Methylene chloride fraction	674.27 528.18 900.32	2488.75 1648.32 5021.33	2.260±0.338	39.3	674.27 528.18 900.32	2488.75 1648.32 5021.33	2.260±0.338	46.4
Ethyl acetate fraction	457.20 357.65 590.54	1705.19 1177.92 3116.66	2.242±0.320	45.3	546.63 432.77 705.31	1889.09 1314.59 3409.95	2.380±0.337	57.2
Butanol fraction	206.91 123.81 295.13	1514.35 880.79 4712.40	1.483±0.302	100.0	312.72 221.63 424.01	1800.25 1119.84 4266.58	1.686±0.284	100.0

Table 2. Toxicity of isolated compounds against larvae and adult females of *T. urticae* after 7-days of treatment.

Fractions	Isolated compounds	Larvae				Adult females			
		LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value
Methylene chloride	p-Hydroxy benzaldehyde (1)	1016.04 603.94 6708.65	5842.19 1887.09 6143.00E+2	1.687±0.532	10.8	1101.26 644.21 8530.98	5854.29 1893.08 7299.29E+2	1.766±0.567	21.6
	3,5-Dimethoxy-4-hydroxybenzaldehyde (2)	1536.96 1125.26 2392.70	8817.61 4675.19 31190.19	1.689±0.304	7.1	1738.95 1265.19 2799.73	9746.64 5072.11 36431.75	1.712±0.313	13.7
	Scopoletin (3)	3834.16 2516.01 5486.59	23152.48 23152.48 56252.40	1.641±0.280	2.9	7943.96 3185.74 1896.53E+1	2902.17 E+2 86773.13 33942.72E+2	0.820±0.163	3.0
Ethyl acetate	Amentoflavone (4)	209.90 137.53 332.94	2336.59 1011.23 18882.46	1.225±0.280	52.3	380.03 276.29 615.36	2290.46 1157.72 9747.82	1.642±0.318	62.5
Butanol	Cosmosiin (5)	120.40 66.41 179.17	1345.72 660.34 7665.67	1.223±0.281	91.1	237.68 169.73 351.52	1716.94 890.89 6905.86	1.492±0.292	100.0
	Anisofolin-B (6)	109.68 60.80 160.56	1073.68 568.08 4657.66	1.294±0.284	100.0	278.59 209.57 394.51	1450.24 844.01 4109.39	1.789±0.312	85.3

respectively.

According to the toxicity assay, it was found that the flavonoid glycosides (5) and (6) were the most effective compounds and considered to be one of the active ingredients followed by biflavonoid compound (4), this is in agreement with the previous studies reported for other flavonoids glycosides isolated from butanol fraction of *Polygonum equisetiforme* which was the most effective fraction against the larvae and adult females of *T. urticae* reported by Dawidar et al. (2014).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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