Effect of Leaf Crude Extracts of Tarchonanthus Camphoratus (Asteraceae), Acalypha Fruticosa (Fabacea) and Tagetes Minuta (Asteraceae) on Fecundity of Phlebotomus Duboscqi

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Abstract

Purpose: *This study was carried out to find out the effects of Tarchonanthus Camphoratus (Asteraceae), Acalypha fruticosa (Fabacea) and Tagetes minuta (Asteraceae) crude extracts on the fecundity of P. duboscqi.*

Materials and Methods*: The extracts were prepared from the dried aerial parts of T. camphoratus, A. fruticosa, and T. minuta. Ten female sand flies were aspirated into vials where they were fed on a mixture of the plant extracts and sucrose solution prepared in a ratio of 1:1.*

Results: *These crude plant extracts were found to reduce the fecundity of P. duboscqi significantly (P< 0.05). The extracts were found to reduce the fecundity of P. duboscqi by 73% (A. fruticosa), 53% (T. minuta) and 26% (T. camphoratus) (P< 0.05).*

Conclusion: *The higher level of A. fruticosa, T. minuta and T. camphoratus activities would potentially reduce the population of sand flies. This study has provided proof of the effects of these medicinal plants on the vectorial capacity of sand flies.*

Key words: Fecundity, *Phlebotomus duboscqi, Tarchonanthus camphoratus, Acalypha fruticosa, Tagetes minuta*

1. Introduction

Visceral leishmaniasis, which is usually caused by *Leishmania donovani*, and cutaneous leishmaniasis, caused by *L.* a*ethiopica, L. major, L. tropica, and L. donovani*, are endemic in Kenya. Visceral leishmaniasis was first reported in Kenya among King's African Rifles troops in Lake Turkana, Southwest Ethiopia in the 1940s (Ngure *et al*., 2009). The disease is transmitted by *P. martini*, though other vectors including *P. orientalis* have been reported. In 2001, there was an outbreak of VL in Wajir and Mandera districts of North Eastern Kenya with 904 patients diagnosed between May 2000 and August 2001 (Marlet *et al*., 2003). Cutaneous leishmaniasis due to *L. major* is transmitted by *Phlebotomus duboscqi* mainly found in animal burrows where it feeds on rodents that are frequently infected. The only proven vector of this *Leishmania* in Kenya is *Phlebotomus duboscqi* Neveu-Lemaire. This sand fly acquires *L. major* infections from several indigenous species of rodent which serve as reservoir hosts.

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Disease transmission can be interrupted by controlling the vectors using various methods. Leishmaniasis experts advocate for vector control especially for areas of anthroponotic transmission (Hailu *et al*., 2005). Vector control measures employed include spraying houses with insecticides where sand flies are endophilic and using treated and untreated bed nets where sand flies are endophagic (Piscopo and Mallia, 2006). Personal protection using repellants and nets is an important aspect. However, the extensive and unbalanced use of chemical insecticides has created problems like enhancing resistance of sand flies to synthetic insecticides pollution of environment and adverse effects on the non-target flora and fauna inhabiting the same aquatic habitat (WHO, 1992). These steadily growing problems demand an intensive search for new products that are environmentally safe, target specific and degradable (Kaushik and Saini, 2008).

Botanicals derived from plants have also been used successfully in controlling the vector and other insect pests. These plant extracts are safe to use and have desirable properties. Plant essential oils have been found useful in controlling sand flies. *Tarchonanthus camphoratus, Acalypha fruticosa* and *Tagetes minuta* plants are locally available in Kenya. They are partially dried and hung indoors to repel biting flies in leishmaniases prevalent areas (Ireri *et al*., 2010). Their extracts have been found to be insecticidal to *P. duboscqi*. However, the mechanism of action of these plants has not been fully investigated. In this study, we sought to assess the effects of *Tarchonanthus camphoratus, Acalypha fruticosa* and *Tagetes minuta* crude extracts on the fecundity of *Phlebotomus duboscqi*.

2. Materials and Methods

2.1 Study site

The study was carried out at the Kenya Medical Research Institute's Centre for Biotechnology Research and Development (CBRD), Nairobi, Kenya between January and April 2011. *Phlebotomus duboscqi* were obtained from KEMRI's insectary. This colony originated from sand flies collected in animal burrows and from termite hill ventilation shafts in Rabai area near the town of Marigat in Baringo district, Rift Valley province in Kenya.

2.3 Study Design

A comparative experimental design was used using the three plant crude extracts. Their activities were compared against each other for their activities against the adult sand flies. The crude extracts were also combined and studied for synergistic effect on the sand flies. The extracts were mixed with sugar bait, dark Corn Karo syrup before feeding the sand flies. All the experiments were carried out using a completely randomized block design

2.4 Collection and preparation of plant materials

Floral and foliar parts of *Tarchonanthus camphoratus, Acalypha fruticosa,* and *Tagetes minuta* were collected from Marigat division, Baringo district, Rift Valley province, Kenya. Botanical identification was carried out with the help of taxonomists from the National Museums of Kenya. All the collected parts of the plants were washed and left to dry completely under a shade for one month and then transported to the laboratory where they were left to dry further under room temperature. The dried specimens were then ground using an electrical mill in readiness for extraction.

2.5 Extraction of plant materials

The sample extraction procedure was carried out as described by Harborne (1994). Briefly, cold sequential extraction was carried out on plant material with analar grade organic solvents of increasing polarity. The solvents used include methanol and ethyl acetate to prepare two different extracts. Three hundred milliliters of methanol were added to 300g of the shred specimen and flasks placed on a shaker and soaked for 48 hours. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 300 ml of methanol for 24 hours until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30 - 35°C. The concentrated extracts were transferred to a sample bottle and dried under vacuum; the weight of the dry extracts was recorded and stored at 4°C until required for bioassay. The process was repeated for ethyl acetate.

2.6 Sand fly colony

The sand fly colony used belonged to the Phlebotomine genus specifically *Phlebotomus duboscqi* Neveu Lemaire. This colony is being reared at the Kenya Medical Research Institute for research purposes.

Phlebotomus duboscqi flies used were collected from Marigat division, Baringo district in rift valley province, Kenya. The colony was established using periodically field captured sand flies and inbreeding. In the insectary, Syrian golden hamsters were used for blood feeding female sand flies for egg development. Blood fed females were aspirated into oviposition vials for egg laying. The insectary has favorable conditions for the development of the sand flies. Temperature was maintained at 25±1°C and a relative humidity of 78-83 %. Adult sand flies were kept in cages where they were fed on slices of apples as a source of energy. Eggs in oviposition vials were left to hatch into larvae that were fed on larval food which consisted of rabbit chow and droppings. All the experiments were carried out using 2-3 day old adult flies.

2.7 Evaluation of Minimum Inhibitory Concentration (MIC)

Ten *Phlebotomus duboscqi* flies were fed on sugar solution mixed with the crude extracts in the ration of 1:1 of several concentrations (1mg/ml to 20mg/ml) of test compounds. Mortality and fecundity was assessed daily by counting dead flies and counting eggs laid on oviposition vials in order to evaluate MIC. The lowest concentration of the samples that killed the sand flies and inhibited their fecundity was considered as the MIC.

2.8 Effects of *T. camphoratus, A. fruticosa,* and *T. minuta* on fecundity of *P. duboscqi*

Ten female *Phlebotomus duboscqi* adult flies, 2-3 days old were placed in plastic vials partially filled with plaster of Paris and fitted with screen tops. Only the blood fed females were aspirated into the plastic vials where they were fed on sugar solutions laced with pure compounds of the extracts. The insoluble compounds were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1%, after this had been assayed as non-toxic and without inhibitory effects on parasite growth, as previously demonstrated (Dorin *et al*., 2001). These extracts were then mixed with Karo dark corn syrup (Best Foods, CPC International, Inc., Englewood Cliffs, NJ), in the ratio of 1:1. The plant extracts were then prepared into 2.5 mg/ml, 5 mg/ml and 10mg/ml concentrations which were used in the experiment. Egg development was observed for 8 days (for longest surviving flies) and the number of eggs laid on the oviposition vials was counted. Dead flies were dissected and their abdomens observed for any eggs retained in the abdomen. Three replicates were used in this experiment. Fecundity was compared with sand flies that fed on dark corn syrup that was diluted with distilled water and 0.1% DMSO (dark corn Karo syrup mixed with distilled water and 0.1% DMSO without extracts).

2.9 Data Analysis

All experiments were carried out in triplicate. The mean and standard deviation of three experiments were determined. Statistical analysis of the differences between mean values obtained for the experimental groups was done by analysis of variance (ANOVA) to analyze the significance of the results and student's *t* test. *P* values of $\leq$ 0.05 were considered significant.

3. Results

The average number of eggs laid by sand flies after exposure to the different crude extracts is shown in fig. 1. From the figure, it is evident that the sand flies that had fed on *A. fruticosa* crude extract did not lay eggs at 10 mg/ml concentration while those that had fed on *T. minuta* crude extract laid 4.00±2.31 (mean±SE) at the same concentration. The sand flies that formed the control group had a mean of 15.00±1.00 (mean±SE). The observed difference was significant (P=0.014). However, egg laying was significantly higher in *T. camphoratus* crude extract (10.67±1.76, mean±SE) than in *T. minuta* crude extract (P=0.002).

Further analysis showed that the number of eggs laid differed significantly in the different methanolic extracts (*P*=0.014). There was also a significant difference among the ethyl acetate crude extracts used (*P*=0.002). The mean number of eggs laid when comparing methanolic and ethyl acetate crude extracts differed with more eggs being laid in the ethyl acetate extracts than in methanol extracts. The means shows significant difference with *A. fruticosa* having the lowest mean (6.50±1.76) while *T. camphoratus* had the highest (12.00±1.42) (fig. 2). Among the ethyl acetate extracts, *T. camphoratus* had the highest number of eggs laid, 10.67 ±1.85 followed by *T. minuta* with 8.67±1.33 and finally *A. fruticosa* 5.33 ±0.33 (mean±SE). However, the difference between methanolic and ethyl acetate crude extracts was not significantly different (*t*=6.597, *P*>0.05).

Further analysis showed that there was a significant difference in the mean number of eggs in the three crude extracts. The flies which died after feeding on the crude extracts were dissected in physiologic saline and their abdomens were found to be full of undigested blood meal.

The crude extract combinations used were *A. fruticosa + T. minuta, A. fruticosa + T. camphoratus* and *T. minuta + T. camphoratus.* The total mean number of eggs laid after feeding on *A. fruticosa + T. minuta* was 7.00±0.57 eggs (mean±SE), *A. fruticosa + T. camphoratus* 9.33±0.67 eggs and *T. minuta + T. camphoratus* 13.60±1.69. The number of eggs per fly was significantly low across all the three combinations used as compared to the control (fig. 3). However, the effect of these combined extracts was not significantly different from those of individual extracts. In both methanol and ethyl acetate extracts, the effects of *A. fruticosa + T. minuta* combination differed significantly ($P<0.001$) from the *A. fruticosa + T. camphoratus* and *T. minuta + T. camphoratus* combinations (Fig. 4).

4. Discussion

Results from this study shows that the crude extracts investigated, significantly depressed the mean number of eggs laid per female fly although this was dose dependent. The result shows that *A. fruticosa,* and *T. minuta* crude extract had a greater effect on fecundity. This may be attributed to the fact that these plants have high concentrations of the active compounds or the flies fed more on these extracts than on *Tarchonanthus camphoratus.* There is evidence that the secondary compounds in *T. minuta* are effective deterrents of numerous organisms, including: fungi pathenogenic on humans, bacteria, round worms in general, trematodes, nematodes and numerous insect pests through several different mechanisms (Mohamad *et al*., 2010).

Studies have shown that egg laying period can stretch up to 15 days post feeding (Mauricio *et al.*, 2010) but in this study the number of eggs recorded was observed on the 7th day. The engorged dead flies were dissected and their abdomens observed for the presence of eggs. It was revealed that their abdomens were still full of undigested blood meal even after 6 days post feeding. It has been reported that decreased fecundity is linked to the quantity of proteins obtained from a blood meal (Volf *et al*., 2001). Harre *et al*., (2001) compared fecundity among sand flies fed on various sources of mammalian blood. His study showed that egg development depends on the blood meal protein. Therefore, the reduced fecundity in this study may be due to the inhibition of blood meal digestion leading to less proteins being used for egg development.

Acalypha fruticosa was found to be more effective in reducing the fecundity of *P. duboscqi* in both methanol and ethyl acetate crude extracts followed by *T. minuta*. *T. camphoratus* had the least effect on fecundity and this may be attributed to the strong smell of the crude extract which might have deterred the flies from feeding on the plant extracts. *T. camphoratus* has insect repellent properties when in combination with a natural or synthetic triglyceride which improves its activities. Other studies have shown that *T. camphoratus* has no antimicrobial activity. However a more recent investigation of antimicrobial activity of aqueous, ethanolic and hexane extracts of dried leaves did not demonstrate *in vitro* inhibitory effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* or *Klebsiella pneumonia (*McGaw *et al*., 2000*).* Combined crude extracts had a significant effect on fecundity when compared to the control although; this effect was not significantly different from the individual crude extracts. This reveals that there is no synergistic effect when the extracts were combined.

The observation that *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* have effect on egg development of *P. duboscqi* has important implications in vector control. This is because the higher level of their activities would potentially reduce the population of sand flies hence reducing leishmaniasis cases. The approach used here may be useful in the application of these plant extracts against Phlebotomine sand flies which are the vectors for leishmaniasis.

5. Conclusion

This study revealed that the plants *Acalypha fruticosa*, *Tagetes minuta* and *Tarchonanthus camphoratus* have a potent effect on the fecundity of *P. duboscqi*. Therefore the plants can be selected for further studies for processing them into insecticides against sand flies.

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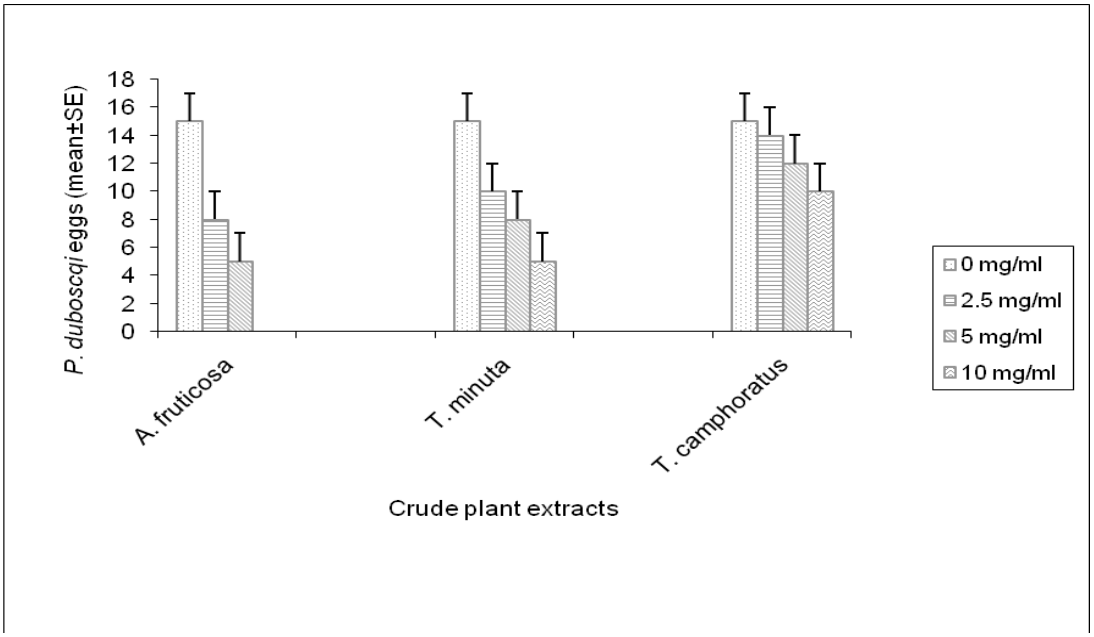

Figure 1: Mean number of eggs laid by *P. duboscqi* after feeding on methanolic crude extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus*

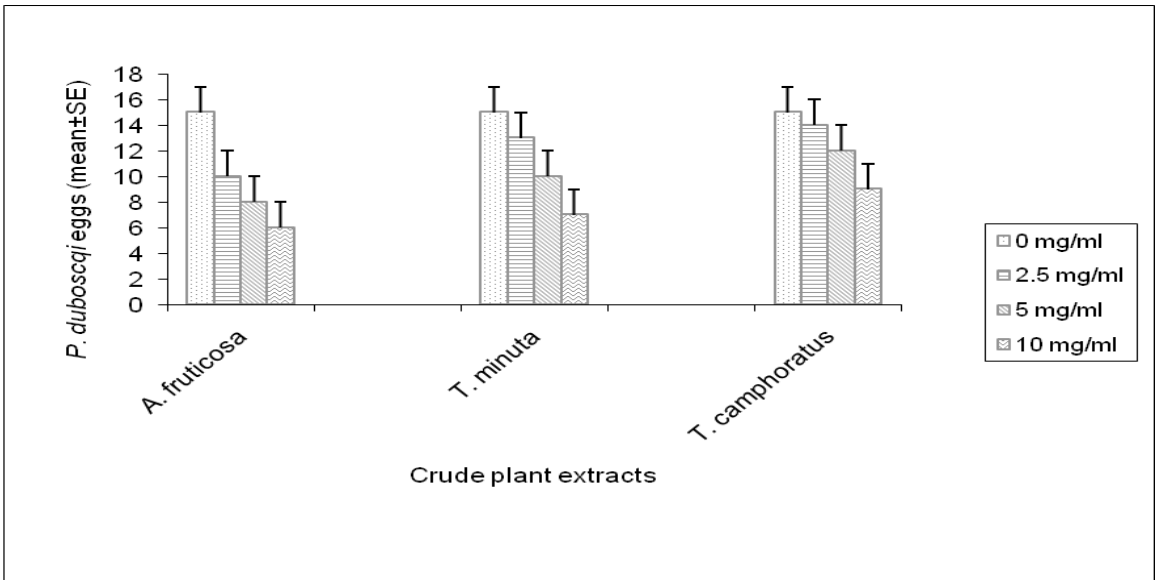

Figure 2: Mean number of eggs laid by *P. duboscqi* after feeding on ethyl acetate crude extracts of *A. fruticosa, T. minuta* and *T. camphoratus*

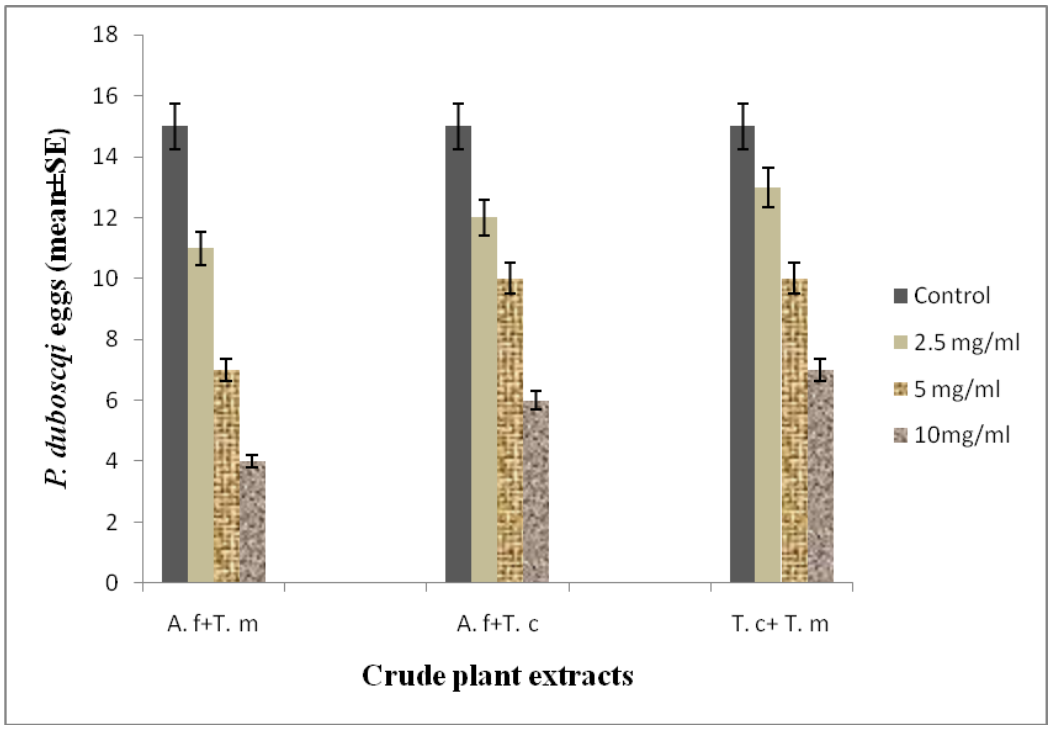

Figure 3: Mean number of eggs laid by *P. duboscqi* after feeding on the combined methanol extracts
Key
A. f + T. m = *Acalypha fruticosa + Tagetes minuta*
A. f + T. c = *Acalypha fruticosa + Tarchonanthus camphoratus*
T. c + T. m = *Tarchonanthus camphoratus + Tagetes minuta*

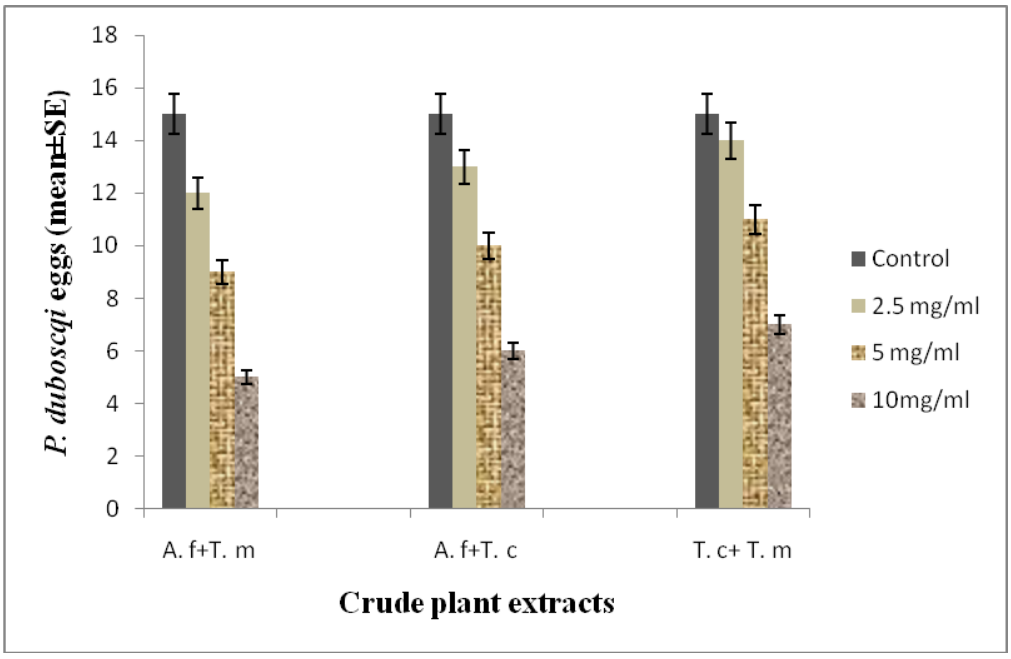

Figure 4: Mean number of eggs laid by *P. duboscqi* after feeding on the ethyl acetate combined extracts
Key
A. f + T. m = *Acalypha fruticosa + Tagetes minuta*
A. f + T. c = *Acalypha fruticosa + Tarchonanthus camphoratus*
T. c + T. m = *Tarchonanthus camphoratus + Tagetes minuta*