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Laboratory Evaluation of Selected Medicinal Plant Extracts in Sugar Baits and Larval Food Against Phlebotomus Duboscqi Neveu Lemaire (diptera: Psychodidae), A Vector for Cutaneous Leishmaniasis in Kenya

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Abstract

Introduction; The efficacy of Tagetes minuta (Asteraceae), Acalypha fruticosa (Euphorbiaciae) and Tarchonanthus camphoratus (Compositae) extracts were evaluated for the control of Phlebotomus duboscqi while incorporated in sucrose. This is the first time plant extracts have been used in sucrose baits against sand flies as opposed to environmentally unfriendly synthetic insecticides.

Materials and methods; The plants were collected from Marigat area, Baringo district in the Rift Valley Province of Kenya, an endemic area for leishmaniases. Extraction was done using N-hexane, dichloromethane, ethyl acetate and methanol.

Results; The extracts showed significant mortality (P< 0.05) to both males and females and had comparable LD50 values in Tagetes minuta and Acalypha fruticosa extracts bioassays. The lowest LD50 value for females was 10.6mg/ml in ethyl acetate bioassays in Tagetes minuta and Acalypha fruticosa extracts, while the highest was 12.0 mg/ml in Tagetes minuta methanol extract. Males had the lowest value of 9.9mg/ml in Tagetes minuta methanol extract, while the highest was in Acalypha fruticosa methanol extract with a value of 15.5mg/ml. Results however showed that there was no significant mortality (P> 0.05) difference between males and females but mortality significantly differed (P<0.05) at various concentrations. Tarchonanthus camphoratus and combined extracts however showed weaker insecticidal properties than from separate extracts. Feeding the larvae using extracts in larval food and plain powders nonetheless revealed no larvicidal properties in the plant samples.

Conclusion; The results showed potent properties and may guide future research initiatives aimed at controlling sand flies using sugar baits as alternative approaches to conventional methods.

Keywords

Acalypha fruticosa Forssk (Euphorbiaciae), botanical compounds, Tagetes minuta Linnaeus (Asteraceae), Tarchonanthus camphoratus L (Compositae), phlebotomine sand flies

Introduction

Leishmaniases refer to a group of primarily zoonotic diseases caused by obligate intracellular protozoan parasites of the order Kinetoplastida, family Trypanosomatidae, genus Leishmania Ross, 1903 [1, 2]. They are among the most neglected and endemic in developing countries affecting predominantly the poorest in society [3] and are associated with about 2.4 million disability-adjusted life years and around 70,000 deaths per year [4]. These constitute important public health problems [5], and are among diseases in dire need of improved control tools [6]. Current approaches to the control of these vector transmitted diseases are characterized by increased resistance of vectors to synthetic insecticides due to development of detoxification mechanisms, the increasing costs of the insecticides, toxicity, soil and water contamination.

Co-evolution has equipped plants with overabundance of chemical defenses against insect predators, and has been harnessed since ancient times to control insect [7]. It is noteworthy that plant based compounds, if meticulously harnessed can be critical sources of ideal control agents since they are safe in most flora and fauna and are target specific based on their mode of application. Moreover, search for botanical compounds with a long-lasting, effective and safe protective activity is as old as human culture [8]. Effectiveness of plant extracts in arthropod control, and especially phlebotomine sand flies has been shown in some product mixtures of essential oils and crude extracts. Rotenon and rotenoids were shown to have insecticidal properties against Lutzomyia

longipalpis Lutz and Neiva [9].

Plant extracts including those of pyrethrum, nicotine and rotenone were among the foremost compounds used to control insects of medical and agricultural importance. Tarchonanthus camphoratus distilled leave extracts, derivatives and formulations were shown to have insecticidal, repellent and medicinal uses. Wild animals are known to rub against leaves to keep off biting insects [10]. The plant is known in Swahili as 'leleshua' in Kenya and its leaves are burnt to repel biting insects. Acalypha fruticosa shrub has been used extensively for its medicinal value, repellent and insecticidal activities against ectoparasites and flies [11]. Tagetes minuta extracts have been shown to be effective against many organisms including mosquitoes [12] and head louse [13]. Bioassay results of natural plant products may vary between same and different plants species but still remain possible alternatives to synthetic substances, as they are effective and compatible with human and animal life and the environment [14]. Although in the past plant extracts have been used in phlebotomine sand fly control, few have probably been evaluated while incorporated in sugars as baits or premixed with larvae food in feeding bioassays.

Aiming at the discovery of cost effective alternatives for the control of disease vectors and especially phlebotomine sand flies, the efficacy of Tagetes minuta, Tarchonanthus camphoratus and Acalypha fruticosa extracts incorporated in sucrose solutions has been evaluated against adults. The extracts were also incorporated in larval food and tested against Phlebotomus duboscqi larvae. These plants are known in local language (Turgen) as "Bangi, lelechuet and lekulu" respectively.

Materials and Methods

The flowering parts and leaves of Tarchonanthus camphoratus, Acalypha fruticosa, and Tagetes minuta were collected from Marigat area of Baringo district in the Rift Valley Province of Kenya. Voucher specimens of the plant parts were preserved at the National Museums of Kenya herbarium. The specimens were dried under the shade and packed in paper bags then transported to Kenya Medical Research Institute leishmaniasis laboratory where further drying was done under shade for a month until completely dry. The dry samples were ground to fine powder using an electric mill. Conical flasks of one litre capacity were used to soak 100 g of samples in 300ml of ethyl acetate. The samples were placed on a shaker for 48 hours, filtered and re-soaked further with 300 mls of the solvent for 24 h until the filtrates remained clear. The extracts were then filtered using Whatman filter paper number 1, concentrated and dried under vacuum using rotary evaporator at 30-35o C [15]. The wet powders were dried under shade and then soaked in methanol and the procedure repeated as above. Universal sample bottles were pre-weighed before the concentrates were transferred into them and then re-weighed and the weight of the dry extracts recorded. They were then stored at -20 °C until required for bioassay.

Sand flies for bioassay

Sand flies used in this study were acquired from an established colony of Phlebotomus duboscqi that were originally captured from Marigat division, Baringo district, Rift Valley, in Kenya and was conserved at the Centre Biotechnology Research and Development insectaries in Kenya Medical Research Institute (KEMRI). The colony was reared and preserved according to methods previously described [16]. Female sand flies were fed on blood using anaesthetized Syrian golden hamsters and reared at 28 ±1oC, and an average RH of 85-95% and 12:12 (L:D) photoperiod in Perspex cages using appropriate Arthropod Containment insectary level guidelines [17]. Sand flies obtained carbohydrates from slices of apple introduced into the rearing cages on daily basis.

Sucrose- extracts feeding technique

This was done in a similar technique as is normally used to feed sugar to contained insects [18], equivalent to the one used to feed sand flies with Bacillus sphaericus in sugar baits [19], but in a laboratory set up using aqueous plant extracts. Phlebotomus duboscqi adult sand flies were carefully aspirated into plastic rearing jars partially filled with plaster of Paris and fitted with screen tops. Ten percent sucrose solution was used to prepare plant extract concentrations of 2.5, 5.0 and 10 mg/ml. Cotton wool pads were soaked in the preparations and placed on the screen tops. Two triplicate series with 10 flies each of P. duboscqi were used for each plant extract and dilution. The first triplicate contained 10 females and the second triplicate contained 10 males in each jar. 60 specimens were assayed for each extract and dilution. In the negative controls, the flies were fed on 10% sucrose solution soaked in cotton wool pads and placed onto the screen tops. Males and females were tested differently and the mean lethal dosage, designated LD50 determined daily.

Larval bioassays

This was done as previously described [20], but with minor modifications. Ten Phlebotomus duboscqi larvae were gently placed into four triplicate series of vials using a camel hair brush wetted in distilled water.

The first triplicate series contained 1st instar larvae, the second triplicate 2nd instar larvae, the third triplicate contained the 3rd instar larvae, and the fourth triplicate contained the 4th instar larvae in each vial. One gram of larval food prepared from a fungal growth obtained from rabbit chow was mixed with each extract solution prepared into preliminary concentrations of 20 mg/ml and let to dry overnight under shade. The vials were appropriately marked for each plant extract. Small amounts of the prepared dry food-extract mixtures were then sprinkled into the vials each day. The four triplicate series of larvae were used for each plant extract. Larvae that fed on larval food mixed with distilled water and dried under the same condition as the treatments were used as controls. Larvae were also fed on plain powdered plant parts and without any larval food mixture. Those that fed on larvae food alone formed the control group. Larvae were monitored daily and mortality recorded for analysis. Therefore, at least 120 larvae were assayed for each plant extract. Mean lethal dosage designated LD50, was determined daily.

Data analysis

Experiments were done in triplicate and data were entered into Microsoft excel program. Control groups in the experimental bioassays with more than 20% mortality were repeated. Data on the effects of the products were subjected to computerized Probit analysis [21] for LD50 values on all bioassays. Variation effects of extracts and between sexes were compared using ANOVA [22]. Values of $P \leq 0.05$ were considered significant.

Results and Discussions

The extracts of Tagetes minuta were established to be more effective in mortality to adults than other extracts (Illustration 1). LD50 values for the females in 48 hours of 12.0 mg/ml ($\chi 2= 13.6$, P= 0.02, mean 39.2%) while male LD50 was 9.9 mg/ml ($\chi 2= 23.7$, P= 0.05, mean 45.8%) in the methanol bioassays. The LD50 for the females in the ethyl acetate assay was 10.6 mg/ml ($\chi 2= 12.7$, P= 0.03, mean 38.3%) while males had LD50 of 10.5 mg/ml ($\chi 2= 10.1$, P= 0.07, mean 37.5%). This plant has been studied by several groups and its active components identified as thiophene derivatives present in many Asteraceae species [7, 23]. Active components previously described for this plant include 5-E-ocimenon [24] and four thiophene derivatives which when assayed displayed an LC50 of 3.9mg/l against Aedes aegypti and Anopheles stephensi 3rd instar larvae [25]. Mortality of the sand flies in this study could be attributed possibly to some of these components.

Acalypha fruticosa extracts exhibited properties almost similar to those of Tagetes minuta at 48 hours but exhibited 90-100% mortality after 96 hours of exposure in 5mg/ml and above. Lower concentrations gave low male mortality even after 96 hours of exposure. The LD50 for the females were 11.2 mg/ml ($\chi 2= 22.0$, P =0.04, mean 30%) and 10.6mg/ml ($\chi 2= 20.4$, P =0.02, mean 33.3%) for methanol and ethyl acetate respectively at 48 hours of exposure. The LD50 values for males was 15.5 mg/ml ($\chi 2= 17.5$, P =0.04, mean 30%) (Methanol) and 12.4 mg/ml ($\chi 2= 21.4$, P =0.02, mean 25.8%) (Ethyl acetate extract). Data on insecticidal evaluation on the above plant is not available in records although it was reported that dried leaves are powdered, soaked in water and the solution applied on animal skin and wound as a repellent or insecticide against ectoparasites and flies [11] probably due to insecticidal properties exhibited by the extract of this plant in the present study.

The extracts of Tarchonanthus camphoratus exhibited weaker properties as compared to the other two plant extracts (Illustration 2). The LD50 for the females was 34.4 mg/ml ($\chi 2= 3.6$, P >0.05, mean 6.67%) while males had a LD50 of 37.7 mg/ml ($\chi 2= 3.85$, P > 0.05, mean 12.5%) at 48 hours of exposure. Mortality in the first 24 hours was low in both male and female bioassays. At 96 hours of exposure, mean mortality was 14 (46.7%) for the males in the 10mg/ml bioassay. Female mean mortality at the same time was 12 (40%). Phytochemical analysis done in the laboratory indicated the presence of tannins, saponins and reducing sugars [26]. In studies on Tarchonanthus camphoratus, it was noted that derivatives and formulations of this plant had repellant activities and medicinal uses [27]. It was observed that distilled leaves of the plant yielded compounds with insecticidal activities and a spray lotion containing 3% Tarchonanthus essential oil was shown to be protective against mosquitoes [10]. Wild animals were observed to rub against leaves to keep off biting insects [28] probably due to insecticidal effects revealed in this study. Feeding sand fly larvae on the plant powder and crude extracts recorded no mortality, however. Combination of the methanol extracts of the three plants had no synergistic effects although mortality of the sand flies were lower than for individual extracts except for Tarchonanthus camphoratus.

Insecticidal effects on male and female sand flies

Results showed that there was no significant mortality difference between male and female sand flies ($F = 0.00$, $P = 0.929$) at 48 hours of exposure considering the fact that the LD50 values were comparable. This

showed that mortality of both female and male sand flies was comparable in similar concentrations of the extracts used. Adult sand fly mortality significantly differed at various concentrations tested ($F = 27.2$, $P < 0.05$) (ANOVA).

The study described here was based on the hypothesis that vectors feed on plant secretions, juice and nectar and may therefore feed on the solutions used. Sand flies readily fed on the extracts used in the study. A previous study demonstrated that sand flies could feed on aqueous sucrose solution containing a larval toxicant Bacillus sphaericus Neide, when sprayed on vegetation cover near burrows and termite moulds [29]. Evidence of sugar feeding was tested in the present study using the cold anthrone test [30] done in the preliminaries. Similar works demonstrated that sand flies feeding on noxious plants juice of R. communis, B. glabra, and S. jasminoides had their lifespan reduced considerably and noted that the causes of death were unknown since these plants belonged to three different families [31]. Similar studies using other plant species shown evidence that leaves extracts were toxic to insects and other organisms [32, 33]. Solanaceous alkaloids are also toxic to insects and a ribosome inactivating protein was found in a Bougainvillea species [34].

This study is in agreement with these findings since the plants contain different components. No literature on Acalypha fruticosa fractionation has been found in previous works and is a good candidate for Phytochemistry to identify the active components. Other Acalypha species have been found to possess insecticidal effects against beetles [35]. Although tannins and saponins have been found in Tarchonanthus camphoratus, they may have been the causes of death of the sand flies or different. Secondary compounds of Tagetes minuta which include monocyclic and bicyclic, monoterpenes, sesquiterpenes, flavanoids, thiopenes, and aromatics [36, 37, 38], have mostly been used against many other organisms except on adult sand flies and larvae. Preliminary assay using concentrations of 0.6mg/ml and below of the crude extracts of both Tagetes minuta and Acalypha fruticosa revealed fecundity destabilization of blood fed females, laying very few eggs which hatched inconsistently when compared to the controls. These are interesting insights into novel control strategies and needs further research to elucidate the active components and their mode of action. Feeding sand fly larvae on the whole plant powder and crude extracts recorded no mortality whatsoever, although they were observed to feed well on the products. The only effect observed was stunting of the instars and time taken from one instar to the other increased between one and three days, but gradually pupated and adult flies consequently emerged. The emerged adult sand flies were of the same morphological features and showed no abnormality from the ones from the main insectary. Phlebotomine sand flies just like other dipteran larvae have a highly alkaline midgut, live in humid habitats and feed on dead and/ or decaying matter without any harmful effects [39], hence finding effective botanical products against immature sand fly stages may be difficult but likely.

Conclusions

Since no ethnobotanical information on these plants has been found related to similar use, our findings suggest, for the first time, their potential insecticidal action in sugar baiting and can be used alongside other methods as an alternative strategy against phlebotomine sand flies. This could be a novel technique which could see well designed bait traps set in crucial sites and may avoid injury to non target organisms altogether. However, much work entailing field evaluation is absolutely necessary to explore their performance and compatibility to the existing integrated vector management programs in Kenya.

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Author contribution

Dr. Willy Tonui- Financial management, scientific advisor, manuscript preparation and review, overall supervision

Dr. Jedida Kongoro- Technical advisor, plant extraction, sand fly rearing and review of the manuscript.

Dr. Peter Ngure- Data entry and management,

experimental bioassays, manuscript review
Mr. Laban Ireri- Plant extraction, sand fly rearing, experimental bioassays, Manuscript Preparation and review

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Illustrations

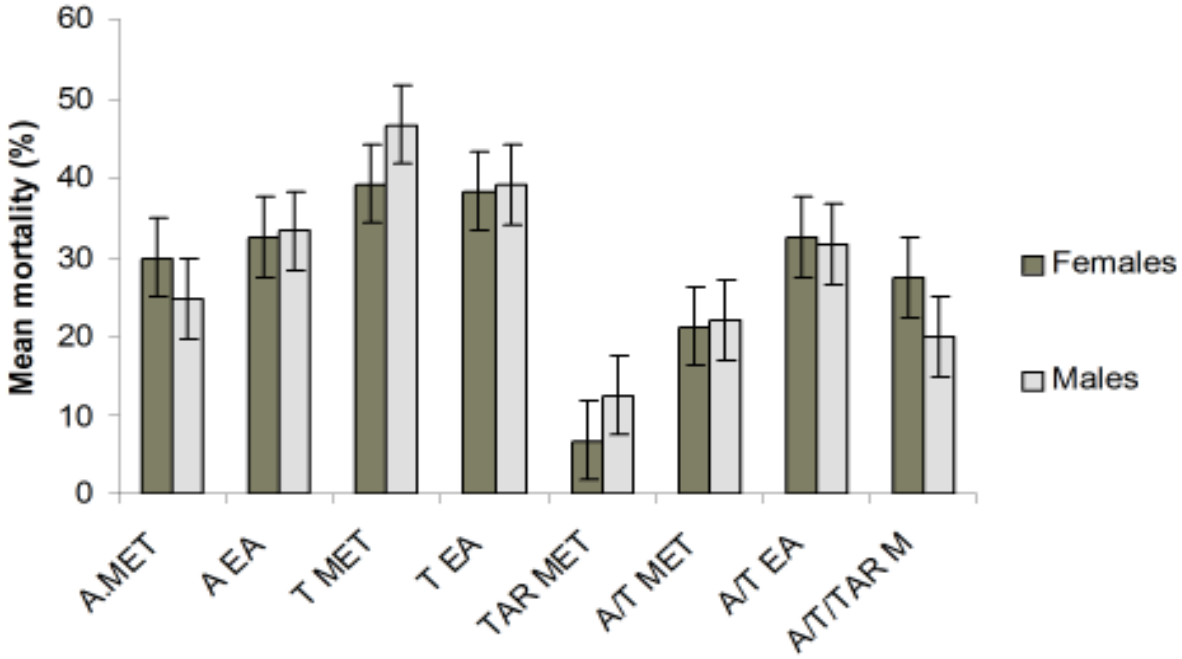
Illustration 1

Comparative effects of the extracts of A. fruticosa, T. minuta and T. camphoratus against adult P. duboscqi incorporated in 10% sucrose solutions at 48 hours of exposure.

Plant	Extract	Sex	LD_{50}[1]	χ^2[2]	*P*
T. minuta	Methanol	male	9.90	23.7	0.051
		Female	12.0	13.6	0.021
	Ethyl acetate	male	10.5	10.1	0.07
		Female	10.6	12.7	0.03
A. fruticosa	methanol	male	15.5	17.5	0.04
		Female	11.2	22.0	0.04
	Ethyl acetate	Male	12.4	21.4	0.02
		Female	10.6	20.0	0.01
T. camphoratus	Methanol	male	37.7	3.85	0.571
		Female	34.4	3.6	0.612
Acalypha/Tagetes	Methanol	male	10.9	40.1	0.01
		Female	9.76	25.3	0.01
	Ethyl acetate	Male	14.7	14.9	0.011
		Female	12.0	13.2	0.021

Illustration 2

Mean percent mortality of adult P. duboscqi exposed to different extracts in sucrose baits at 48 hours of exposure. Thirty sand flies were used in each sex and extract bioassay.



A. MET= *Acalypha* methanol
A. EA= *Acalypha* ethyl acetate
T. MET= *Tagetes* methanol
T. EA= *Tagetes* ethyl acetate
TAR. MET=*Tarchonanthus* methanol,
A/T. MET= *Acalypha/Tagetes* methanol
A/T.EA=*Acalypha/Tagetes* ethyl acetate,
A/T/TAR.M=*Acalypha/Tagetes/Tarchonanthus* methanol

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