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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING, CYTOTOXICITY STUDIES AND LARVICIDAL ACTIVITY OF HEXANE EXTRACT OF *LIPPIA KITUIENSIS*AGAINST *RHIPICEPHALUS APPENDICULATUS*

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ABSTRACT

Ectoparasites particularly ticks are responsible for severe losses in the livestock industry. This study evaluated the larvicidal properties of hexane extract of*Lippiakituiensis*against larvae of *Rhipicephalus appendiculatus*. Contact toxicity was used in the bioassay and mortality data was collected at 6, 12, 24 and 48 hrs.The data obtained during bioassay was then subjected to probit regression analysis to estimate concentration dependent mortality for LC_{50} and LC_{90} values in mg/ml.The LC_{50}in mg/mlwere 12.6(11.0-14.1), 10.6 (9.0-12.0), 6.7 (5.2-7.9), and 4.8(2.2-5.9) while the LC_{90}in mg/ml were 19.5(17.0-24.4), 17.4(15.0-22.0), 10.8 (9.1-14.3) and 7.7 (6.2-13.0) at 6, 12, 24 and 48 hrs respectively.Results of one way ANOVA showed significant difference (P= 0.03, 95%) in activity against the larvae by the hexane extract of *L. kituiensis* at 6, 12, 24 and 48 hrs. Phytochemical screening of hexane extractrevealed presence of saponins, flavonoids, steroids, terpenoids and cardiac glycosides.The activity observed in this extract was attributed to the presence of these phytochemicals. The plant demonstrated no cytotoxicity against vero cells at 500µg/ml, hence the extract was considered safe for practical use in controlling *R. appendiculatus* infestation in livestock.

INTRODUCTION:

Vector borne diseases are a global problem that hinders productivity of animals.These vectors are ectoparasites which comprise ticks, insects and mites. Ectoparasites particularly ticks are responsible for severe losses in the livestock industry. Studies by Wall (2007) found that 80% of 1, 200 million cattle were at risk of getting tick-borne diseases causing a global annual loss of US $ 7,000 million. In Kenya, annual loss due to East coast fever alonewhich is transmitted by *R. appendiculatus*tick was US$ 54.4 million in 2003 (Minjauw and Mcleod, 2003).

R. appendiculatus is a three host ixodid tick and is a vector of major economic importance in Africa. It transmits *Theileria parva* which causes East coast fever a threatening disease in the livestock industry in Eastern, South-eastern and Central Africa. It also transmits corridor disease in cattle, Nairobi sheep disease virus and Thogoto virus to livestock (Walker*et al.,*2000). The use of synthetic chemical acaricidestocontrol ticks in cattle is expensive, sometimes ineffective due to development of resistance and it leads to environmental pollution (Gromboni *et al.,* 2007).

Plants produce novel compounds of medicinal importance. About 28% of all new chemicals launched on to the market between 1981 and 2002 were of plant origin (Newmann*et al.,* 2003), while 24% were artificial products that mimic natural products (Newmann *et al.,* 2000).There are huge prospects in the use of extracts of plants from tropical and subtropical regions of Africa, Asia and South America, to come up with acaricides that can decrease the cost of tick control (Habeeb, 2010).

Lippia (*Verbenaceae*) plant extracts have been used as remedy for gastrointestinal and respiratory disorders in tropical Africa, South and Central America besides being reported to have antimalarial, spasmolitic, sedative, hypotensive, and anti-inflammatory effects (Pascual *et al.,* 2001). Camphor isolated from *L. kituiensis* essential oil was found to have a strong repellent activity against maize weevil compared with NN-diethyltoluamide (DEET) (Mwangi, 1992). *Lippiajavanica* and *Lippiagraveolens* are among the*Lippia* species whose acaricidal efficacy have been ascertained. *L. javanica* aqueous leaf extracts at 10% and 20% w/v were found to be effective at controlling *R. appendiculatus, Rhipicephalus evertsi, Boophilus decoloratus* and *Hyalomma* species (bont-

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legged ticks) at study site in Zimbabwe (Madzimure *et al.,* 2011).*L. graveolens* was found to cause 90-100% mortality on 10 day *Rhipicephalus microplus* tick larvae (Martinez-Velazquez *et al.,* 2011). So far, the acaricidal efficacy of *L. kituiensis*has not been tested and this study aims at determining effect of hexane extract of this plant on*R. appendiculatus*larvae.

MATERIALS AND METHODS:

Sample collection:

Leaves of *L. kituiensis* were collected from botanical-garden of Egerton University in Kenya which is at an altitude of 2,127 meters above sea level. A voucher specimen was deposited at the department of biological sciences, Egerton University.

Extraction of hexane extract:

The Leavesof*L. kituiensis* were air-dried under a shadefor a period of 7-14 days while turning them periodically to expose all the leaves to air. They were weighed frequently until a constant weight was obtained and thus considered dry. Dried leaves were grounded using a blending machine (Thomas-Wiley Laboratory Mill Model 4) at Kenya Agricultural Research Institute (KARI), Njoro. The powdered material was weighed with each 500 g being extracted with 1.8 liters of 95% methanol at room temperature for 72 hrs. It was then filtered through a buchner funnel and the filtrate was concentrated to dryness under reduced pressure using rota-vapor machine (BUCHI – R 205). The concentrated crude methanol extract was placed in a separating funnel then suspended in distilled water to remove available sugars. It was then followed by sequential extraction with ethyl acetate and hexane repeatedly until both ethyl acetate and hexane werecolorless, meaning they were no more compounds present in the methanol extract that could be extracted by both hexane and ethyl acetate. The hexane extract carried least polar phytochemicals while the ethyl acetate extract carried medium polar phytochemicals. Both hexane and ethyl acetate extracts were concentrated to dryness under reduced pressure using rota-vapor machine mentioned in methanol resulting in hexane and ethyl acetate crude extracts.

Larval bioassay:

The larvae used for the bioassay were reared according to (Bailey, 1960) while acaricidal bioassay was done using contact toxicity according to FAO, (2004). Larval mortality data was obtained at 6, 12, 24, and 48 hrs from the start of the experiment.From preliminary experiments, the stock solution of hexane extract was 25 mg/ml while the ethyl acetate extract did not show any larvicidal activity even at high concentration of 25 mg/ml at 6, 12, 24 and 48 hrs hencewas dropped from the experiment.Serial dilution done on hexane stock solutionresulted in 10 concentrations ranging from 25 mg/ml to 5 mg/ml. The concentrations were sprinkled using a pasture pipette on the petri dishes that hadwhatman No. 1 filter paper (15 cm) attached to the bottom using double sided cellophane tape, and contains 20 larvae. During sprinkling, the filter papers were ensured wet, and the larvae were exposed to the sprinkled extracts. The experiment was replicated three times and petri dishes held at 75 % relative humidity at 25°C.The larvae were considered dead if they couldn't move their appendages when prodded with a pin. A negative control was set consisting of 2% DMSO while a positive control was also set consisting of 0.2% v/v of amitraz.

Phytochemical tests:

Chemical tests to identify phytochemical constituents of hexane extract of *L. kituiensis* were carried out. It was done qualitatively, using standard procedures according to (Edeoga *et al.,* 2005; Khan *et al.,* 2011). The tested phytochemicals were tannins, phlobatanins, saponins, flavonoids, steroids terpenoids and cardiac glycosides.

Cytoxicity assay:

Hexane extract of *L. kituiensis* was tested for *in vitro* cytotoxicity using MTT calorimetric assay (Mosmann, 1983). Vero cells (ATCC CCL-81) established from the kidney of a normal African green monkey (*Cercopithecus aethiops*), were used to determine the cytotoxicity of the plant extracts. These cells were obtained from KEMRI Nairobi. The Cells were first grown in Minimum Essential Medium (MEM) Eagle's Base supplemented with 15% Fetal Bovine Serum (FBS), 2.62 g/L $NaHCO_3$, 20 mM L-glutamine, 10 ml/L Penstrep and 0.5 mg Fungizoid using T-75 culture flask.

Culturing of the cells was done at 37^{o}C in 5% CO_2 for 24 hrs and once they attained confluence they were harvested by trypsinization and pooled into 50 ml vial. Cell suspension (1 x 10^5 cell/ml) approximately 100 µl were seeded into the 96-well flat-bottomed micro-titer plate containing100 µl of MEM (growth media) and incubated at 37^{o}C in 5% CO_2 for 48 hrs, to attain confluence. Once confluency was attained, the growth media was aspirated and replaced with 100 µl of maintance media. The cells were then exposed to increased concentrations of the hexane extract ranging from (500 µg/ml to 0.23µg/ml). The contents in the plate were further incubated at 37 ^{o}C for 48 hrs.

After the incubation period, MTT (10 µL of 5 mg/mL) was added into each well and the cells incubated for another 4 hrs until purple precipitates (formazan) were clearly visible under a microscope. Subsequently, the supernatant was removed and replaced with acid-isopropanol (0.04N HCl in isopropanol). The well plate

was gently shaken for 15 minutes to dissolve the formazan, followed by measurement of optical density (OD) using ELISA scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 690 nm. The 690 nm was the absorbance of background reference filter while the 562 nm was the absorbance of formazan. Percentage growth inhibition at each concentration was automatically calculated using a graphic program Ms excel2003, sing the formular below (Ngeny *et al.,*2013).

$$\% \text{ growth inhibition} = 100 - \frac{(\text{OD sample } 562 - \text{OD } 690)}{\text{OD control } 562 - \text{OD } 690} X100$$

The IC_{50}, which is the concentration of the extracts, that reduced viable cell by 50%, was automatically calculated from graphs generated by the graphic program. Extract was consideredcytotoxicy if the IC_{50} < 20 µg/ml according to guidelines set by the National Cancer Institute (NCI) (Geran *et al.,* 1972).

Statistical analysis:

The mortality data obtained was subjected to Probit regression analysis to calculate concentration dependent mortality for the LC_{50} and LC_{90} values and the associated 95% confidence interval. The significant difference in by the extract at 6, 12, 24 and 48 hrs was analyzed using one way ANOVA (Analysis of Variance).

RESULTS:

Larval bioassay:

Mean percentage larval mortalities at 6, 12, 24, and 48 hrs, together with LC values are shown in Table 1. LC_{50} in mg/ml were 12.6(11.0-14.1), 10.6 (9.0-12.0), 6.7 (5.2-7.9), and 4.8(2.2-5.9) while the LC_{90} in mg/ml were 19.5(17.0-24.4), 17.4(15.0-22.0), 10.8(9.1-14.3) and 7.7(6.2-13.0) at 6, 12, 24 and 48 hrs respectively. Hexane extract of *L. kituiensis* gave 100% mean larval mortality at the highest concentration of 25 mg/ml at 6 hrs. This was different from positive control (amitraz 0.2% v/v ®) which gave similar percentage mortality at 48 hrs. The negative control used was 2% of DMSO which showed no activity against the larvae within 0 to 48 hrs. The results of one way ANOVA showed there was significant difference in activity of the extract at 6, 12, 24 and 48 hrs against the larvae P = 0.03 at 95% confidence.

Phytochemicals observed:

Among the tested phytochemicals, those presentin the hexane extract were saponins, flavonoids, cardiac glycosides, steroids and terpenoids.

Cytotoxicity assay:

Growth inhibition of vero cells by hexane extractof *L. kituiensis*is presented in Figure 1.The extract demonstrated no cytotoxic activity against vero cells at 500 ug/ml, as the IC_{50} could not be calculated at this concentration.

Table 1: Mean larval mortalities (%) of hexane extract of *L. kituiensis* between 0-48 hrs

Concentration in mg/ml	Mean larval % mortalities at the hrs shown below			
	6	12	24	48
5	0±0	6.7±5.8	30±10	60±10
7.5	13.3±11.5	20±10	50±17.3	80±20
10	26.7±5.8	43.3±15.3	80±10	100±0
12	40±17.3	56.7±5.8	96.7±5.8	100±0
14	50±10	60±20	100±0	100±0
16	70±17.3	83.3±15.3	100±0	100±0
18	86.7±11.5	96.7±5.8	100±0	100±0
20	93.3±5.8	100±0	100±0	100±0
23	100±0	100±0	100±0	100±0
25	100±0	100±0	100±0	100±0
Amitraz ® (0.2% v/v)*	0±0	56.7±11.5	90±10	100±0
(2% DMSO)†	0±0	0±0	0±0	0±0

*Positive control, †Negative control

P=0.03

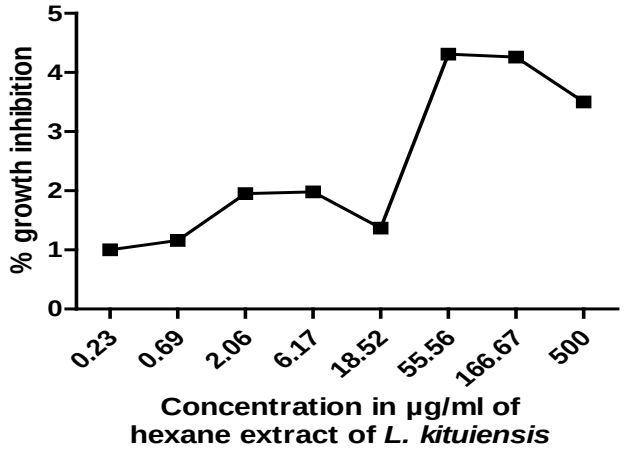

Figure 1: Growth inhibition (%) of vero cells against concentration in µg/ml of hexane extract of *L. kituiensis*

DISCUSSION:

Hexane extracts of different plants have been studied for their acaricidal activity.Leaf hexane extracts of *Vitex Negundo* has been shown to be active against the larvae of *R. microplus* with LC_{50} of 167.20 and LC_{90} of 1875.50 ppm (Kamara *et al.,*2010).Hexane extract of *C. serrate* was also found to be toxic against larvae of *B. Microplus* killing 100% of the *B. microplus* larvae at the concentrations of 50, 25, 12.5, and 6.25 mg/mL after 48 hr (Ribeiro *et al.,* 2008). In this study, hexane extract of *L. kituiensis* showed acaricidal activity against *R. appendiculatus* larvae with LC_{50} in mg/ml of 12.6(11.0-14.1), 10.6(9.0-12.0), 6.7(5.2-7.9), and 4.8(2.2-5.9) while the LC_{90} in mg/ml were 19.5(17.0-24.4), 17.4(15.0-22.0), 10.8(9.1-14.3) and 7.7(6.2-13.0) at 6, 12, 24 and 48 hrs respectively.The activity of observed in the hexane extract of *L. kituiensis*could be attributed to the synergestic effects of the phytochemicals that were present in the extract (Akın, 2010). This is because the phytochemicals observed in this extract have previously been reported to possess acaricidal activity.

According to Shang *et al.,* (2013), flavonoids were the active compounds of acetic ether extract and contributed to the acaricidal activity of *A. coerulea* against *Psoroptes cuniculi*. Terpenoids which include eugenol, isoeugenol and methyl eugenol and butylide nephthalide present in many plant extracts have been shown to possess acaricidal activity against *T. putrescentiae* mite (Kwon and Ahn, 2002). Acaricidal properties of extracts from the aerial parts of *Hipericum polyanthemum* on the cattle tick *Boophilus microplus* were attributed to terpenoids (Ribeiro *et al.,* 2007). Cardiac glycosides isolated from *Calotropies procera* has been shown to be potent against camel tick *Hyalomma drometarii* as indicated by its lower LC_{95} value of 2539 (2207-2922) mg/l compared to Azadirachtin and neem oil which both had LC_{95} value of over 5000 mg/l (Al-Rajhy *et al.,* 2003).Acaricidal activity of root extracts of *P. decandra* against *Tetranychus cinnabarinus* spider mite was attributed to isolated Esculentoside which was the dominant active triterpene saponin (Ding *et al.,* 2013).

The difference observed in larval mortality in both positive control and hexane extract of *L. kituiensis* could be due to difference in concentrations of the active ingreadients. Beside, hexane extract that had several phytochemicals all reported to have acaricidal properties inprevious studies. This differed from the positive control which had a single active ingreadient which is amitraz.

Cytotoxicity results indicated that hexane extract wasnoncytotoxic as indicated by the IC_{50} value which could not be calculated at concentrations used. For cytotoxicity to be observed, the extracts ought to have a concentration of more than 500 µg/ml.Cytotoxicity results of *L. kituiensis* are comparable to previous cytotoxicity studies on phylogenetic related plant *Lippia multiflora* which showed *L. multiflora* tea infusion were not toxic to vero cells and fibroblast cells (Terblanché, 2000). Generally,the extract exhibited selective toxicity, by being toxic to the larvae of *R. appendiculatus* and not to vero cells. Thus it is a potential lead compound for development of plant based acaricides based on its activity and safety.

CONCLUSION:

Results of this study indicate that hexane extract of *L kituiensis* plant have acaricidal properties and could be used to control larvae of *R. appendiculatus* and related ticks.

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