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In vitro anthelmintic effects of crude aqueous extracts of *Tephrosia vogelii, Tephrosia villosa* and *Carica papaya* leaves and seeds

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The prevalence of anthelmintic resistance and the consumer demand for alternative farming systems that limit the use of chemical anthelmintics has made the search for alternative gastrointestinal nematode parasites control methods crucial. Traditional medicinal/herbal plants can offer an alternative to the reliance on chemical anthelmintic drugs. This study evaluates the efficacy of crude aqueous extracts of *Tephrosia vogelii* Hook., *Tephrosia villosa* Pers., and *Carica papaya* Linn. leaves and *Carica papaya* Linn. seeds against gastrointestinal nematodes using *in vitro* egg hatch and larval development inhibition assays. Rectal faecal samples from sheep were subjected to parasitological examination for faecal egg counts (FEC) using the McMaster counting technique. 100 g of dried and poultice aqueous leaf extract of *T. vogelii, T. villosa*, *C. papaya* leaves and seeds was blended into liquefaction in 200 ml of distilled water then boiled at 90-100°C for 1 h and cooled. Levamisol and distilled water were used as positive and negative control in the bioassay. Egg hatch assay revealed more than 95.8% reduction in egg hatch at concentration of 500 mg/ml for dried and poultice paste of *T. vogelii* leaves and *C. papaya* seeds. Larval development inhibition assay results showed that both dried and poultice paste of *T. vogelii* leaves and *C. papaya* seeds extract yielded more than 98% inhibition at a concentration of 500 mg/ml. Based on the LD_{50} dried extract of *C. papaya* seeds was most potent extracts for the inhibition of both egg hatching (49.94 mg/ml) and larval development (49.32 mg/ml). Both poultice and dried extract for all the plants showed significant and dose dependent egg and larval development inhibition. These findings indicate that the evaluated plants have potential anthelmintic effect and could provide viable alternatives for the control of gastrointestinal helminthes in ruminants.

Key words: Aqueous extracts, Anthelmintic activity, Medicinal plants, *Tephrosia vogelii*, *Tephrosia villosa*, *Carica papaya*.

INTRODUCTION

Helminthosis adversely affect ruminants productivity and welfare in both organic and conventional systems (Silva et al., 2011; Chartier and Paraud, 2012; da Silva et al., 2013). It is ranked the highest animal health constraint to

the poor especially in tropical and sub-tropical countries (Perry et al., 2002). There are a number of approaches used to control helminthes in livestock, including nutritional, immunological and biological interventions (Jackson and Miller, 2006). However, most farmers rely on chemical anthelmintic drugs. The cost and non-availability of synthetic anthelmintic for some farmers, emergence of drug resistance, environmental pollution and toxic chemical residues reported in foods derived from livestock are a major cause of concern for many consumers (Jackson and Coop, 2000; Kaplan, 2004; Saddiqi et al., 2010; Sutherland and Leathwick, 2011). Therefore naturally occurring plants with anthelmintic properties could offer alternatives that can overcome some of these problems.

Consumers demand more natural and higher quality foods (Casemiro and Trevizan, 2009). This is due to the growing demand for healthy foods for the people and awareness of impact of chemical residues on the environment. There is a worldwide debate about the development of sustainable food production systems, adapted to different farming conditions. Alternative concepts of agroecology and holistic agriculture, that advocate for the use of integrated management strategies, such as target selected treatment, herbal medicine, and the application of other parasite control alternatives, are undergoing resurgence because of their more sustainable appeal (Molento, 2009). Use of medicinal plants could offer possible alternatives that may be important for agroecological production systems, organic or biological - dynamical systems where the use of chemical drugs is limited (Peixoto et al., 2013).

Tephrosia vogelii Hook. is widely used across Africa as a fish poison, pesticide and for soil enrichment (Neuwinger, 2004; Mafongoya and Kuntashula, 2005; Sirrine et al., 2010; Kamanula et al., 2011). The methanolic leaf extracts have shown anthelminthic activity against *Nippostrongylus braziliensis* (Edeki, 1997). *Carica papaya.* Linn is popularly used as a dessert or processed into jam or wine, while the green fruits are cooked as vegetables (Samson, 1986; Nakasone and Paul, 1998). *C. papaya* is among the 13 plant species used by farmers as anthelmintics to combat worm infestation in livestock in Nigeria (Adedapo et al., 2002). Aqueous extracts of papaya seeds have shown anthelmintic activity against *Haemonchus contortus*, *Trichostrongylus* spp., *Strongyloides* spp. and *Ostertagia* spp. in Sheep (Ameen et al., 2010). Kermanshai et al., (2001) identified benzyl isothiocyanate has the predominant or sole anthelmintic agent in papaya seed extracts against *Caenorhabditis elegans*. Among these botanical species, *C. papaya* (pawpaw) may be preferred as an ethnoveterinary remedy in this part of the tropics because of its adaptability, agro-ecological considerations and availability (Mundy and Murdiati, 1991).

There is, however, no scientific evidence for the anthelmintic effects of *Tephrosia villosa* Pers. The plants were selected and evaluated based on the indigenous knowledge information about their use by farmers against helminthes. The plants are also distributed widely in Kenya and an assessment of their possible efficacies was considered to be of interest.

The study was conducted to evaluate the *in vitro* anthelmintic activity of aqueous extracts of *Tephrosia vogelii* Hook., *Tephrosia vellosa* Pers. and *Carica papaya* Linn. leaves and *Carica papaya* Linn. seeds to validate their use in ethnoveterinary medicine among some farmers in Kenya. These tests are based on the hypothesis that an anthelmintic activity observed *in vitro* would be indicative of a potential *in vivo* activity.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves of *T. vogelii* and *C. papaya* were collected on May 2013 at the Kenya Agricultural Research Institute - National Research Laboratory (NARL) in Nairobi while fresh leave of *T. villosa* were collected from the Kenya Agricultural Research Institute station in Kiboko. *C. papaya* Linn. seeds were collected from ripe pawpaw fruits and washed with clean water to remove dirt. The plants were identified and authenticated in the Department of Botany at the National Museums of Kenya, Nairobi and voucher specimens of each species were deposited at the University of Nairobi herbarium.

The plant materials and seeds were divided into two samples for each plant species. The first samples were ground soon after collection to make a poultice paste of 100 g which was blended into liquefaction in 200 ml of distilled water then boiled at 90-100°C for 1 h and cooled. The second set of samples were dried in shade at ambient temperature for 14 days, ground and milled to powder by electrical blender.

100 g on the powder was also blended into liquefaction in 200 ml of distilled water boiled at 90-100°C for 1 h and then cooled. Both samples were then centrifuged at 1500 rpm for 5 min. The supernatant was filtered through sterile filter papers and stored at 4°C in dark tightly closed glass bottles until used. One millilitre of the filtrate contained 0.5 g (500 mg/ml) of the extract.

Preparation of serial dilutions of aqueous extracts

Serial dilutions of stock solution were performed to yield 10 ml each of 500, 250, 125 and 62.5 mg/ml concentrations of the extract.

Recovery and preparation of eggs

Fecal materials (pellets) were collected per rectum from sheep with natural acute/sub-acute parasitic gastroenteritis due to mixed nematode species. The samples were placed into labeled specimen

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bottles and transported to the Laboratory at the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi. All the samples collected were processed on the same day.

Fecal samples were examined for helminthes eggs using the modified McMaster technique described by Hansen and Perry (1994). Briefly, approximately 3 g of feces were placed in a beaker and 45 ml of floatation fluid (saturated sodium chloride solution) added. The feces were broken into pieces and mixed by stirring with a wooden spatula. The mixture was sieved using a tea strainer into another beaker and subsample taken from using Pasteur pipette while stirring. A McMaster counting chamber was filled with the subsample and the number of eggs counted under a microscope at X40 magnification.

Egg hatch assay

The *in vitro* egg hatch assay method described by Coles et al. (1992) was adopted. A suspension of 20 µl was distributed in three 96-flat-bottomed microtiter plates containing approximately 100 fresh eggs per well and mixed with the same volume of plant extract having different concentrations (500, 250, 125 and 62.5 mg/ml). Four other similar replicates of the plates were made to evaluate the effect of the plant extracts over a three day period. In the control plates levamisol and distilled water was added to the egg suspension. Levamisole was used only at one dose level of 3.125mg/ml as a reference drug. The eggs were incubated in this mixture for 48 h at 27°C and 70% relative humidity. After 48 h, a drop of Lugol iodine solution (Reidel de Hae) was added to stop the eggs from hatching. Hatched larvae (dead or alive) and unhatched eggs were then counted under dissecting microscope.

An inhibition percent (%) of egg hatching was calculated for each extract concentration using the following modified formula of Coles et al. (1992):

$$\text{Inhibition (\%)} = 100 \times (1-X_1)/X_2$$

Where, X_1 is the number of eggs hatched in test extracts, and X_2 is the respective number in distilled water control.

Larval development and viability assay

The procedure used was a modification of the technique described by Hubert and Kerbouef (1992). Aliquots of 150 µl of a suspension with about 100 eggs per well and 20 µl of filtrate obtained by faecal washing during egg recovering were distributed to wells of a 96-well flat-bottomed microtiter plates. This suspension was supplemented with 30 µl of the nutritive medium described by Hubert and Kerboeuf (1984) and comprised of Earle's balanced salt solution (Sigma) plus yeast extract (Sigma) in saline solution (1 g of yeast extract/90 ml of saline solution) at a ratio of 1:9 v/v. The plates were incubated at 27°C and 70% relative humidity. After 48 h, 200 µl of the plant extracts at same concentrations as mentioned above, levamisol and distilled water (control) were added to respective plates. There were four replicates for each extract concentration and control. The plates were further incubated for five days (total of seven days), further development was stopped by addition of one drop of Lugol`s iodine solution. All L1 and L3 larvae in each well were counted under a dissecting microscope. The percentage of development was calculated as the ratio: number of L3/total number of larvae. The percent mortality was calculated from an average of the four replicates.

Statistical analysis

The data from egg hatch assay/test and larval development assay/-test were transformed by probit analysis against the logarithm of extract concentration using SPSS for Windows version 16.0. The extract concentration required to inhibit 50% (LD_{50}) egg hatching and 50% (LC_{50}) larval development were calculated after correction for natural mortality by probit analysis. The comparisons of mean percentage of egg hatching and larval development inhibition at different concentrations with the control, was done by one way analysis of variance (ANOVA). All statistical analyses were performed by SPSS version 16.0 for windows. The post hoc statistical significance employed was the least square difference (LSD), the difference between the mean were considered significant at $P < 0.05$.

RESULTS

Egg hatch assay

The result show that the crude aqueous extracts of the experimental plants inhibited egg hatch of gastrointestinal nematodes at different concentrations as shown in Table 1. At concentrations of 500 ml/ml, poultice paste of *C. papaya* seeds and *T. vogelii* leaves and dried leaves of *T. vogelii* and *C. papaya* seeds showed efficacies greater than 95%. The LC_{50} for egg hatch inhibition were highest for dried and poultice paste of *C. papaya* seeds as shown in Table 3. Both dried and poultice paste of *C. papaya* leaves showed the lowest egg hatch inhibition among the extracts. Very low effects were recorded for distilled water control group. Increasing the concentration of the extracts caused a dose dependent significant ($P < 0.05$) decrease in egg hatch for all the extracts tested.

Laval development inhibition

The average efficacy of the decoctions to inhibit larval development is show in Table 2. The larval development inhibition of poultice and dried *C. papaya* and *T. Vogelii* were higher than 98% at concentration of 500 mg/ml. There was no significance difference between poultice and dried *C. papaya* and *T. Vogelii* for larval development inhibition at 500 mg/ml ($P > 0.05$). The minimum larval development inhibition was recorded for distilled water with a mortality rate of 1.3±4.03%. The LC_{50} for larval development are shown in Table 3.

Both poultice paste extract and dried extracts showed a dose dependent activity against both egg inhibition and larvae development inhibition for gastrointestinal nematodes. However, the overall performance of the dried extracts was better than that of the poultice paste extracts of the same extract.

DISCUSSION

This study demonstrates the existence of biologically active compounds with ovicidal and larvicidal effects in the plant extracts on gastrointestinal nematodes, even after heating for 1 h. The lower activity of dried and

Table 1. The mean inhibition of egg hatching ± SD for the different plant extracts compared to the distilled water negative control and Levamisol (3.125 mg/ml) positive control.

Concentration (mg/ml)	Egg hatching (%) ± SD					
	***C. papaya* leaves**	***C. papaya* seeds**	***T. vogelii* leaves**	***T. villosa* leaves**	**Levamisol**	**Distilled water**
Poultice paste (set 1)						
500	57.8±3.30	99.5±0.58	95.8±1.71	93.2± 0.9		
250	28.0±6.06	83.5±1.29	68.3±2.22	67.3±2.99		
125	16.0±4.97	72.3±4.57	52.3±4.19	55.5±4.44		
62.5	6.2±3.26	58.7±5.03	36.7+5.05	36.1±5.54		
31.25	3.4±2.19	37.8±4.11	24.9±4.12	21.3±2.10		
					100.00	1.3±2.03
Dried (set 2)						
500	59.5±3.42	99.0±0.82	95.8±1.71	87.0±3.16		
250	32.3±2.99	82.5±2.89	77.8±2.36	60.3±4.57		
125	19.8±5.06	75.3±3.78	66.3±3.60	47.8±2.99		
62.5	9.9±2.47	62.1±3.90	41.3±3.90	28.4±4.97		
31.25	6.9±2.62	35.5±4.79	29.7±6.88	19.9±4.29		

SD = Standard deviation

Table 2. The mean larval inhibition ± SD for the different plant extracts compared to the distilled water negative control and Levamisol (3.125 mg/ml) positive control.

Concentration (mg/ml)	Larval Inhibition (%) ± SD					
	***C. papaya* leaves**	***C. papaya* seeds**	***T. vogelii* leaves**	***T. villosa* leaves**	**Levamisol**	**Distilled water**
Poultice paste (set 1)						
500	63.0±2.58	99.5±0.58	99.0±1.41	81.8±2.99		
250	39.5±4.04	78.8±2.22	78.8±2.50	52.8±3.86		
125	23.3±3.59	71.8±2.50	69.3±2.63	41.0±3.65		
62.5	14.9±4.87	54.6±4.92	52.1±4.34	33.4±3.18		
31.25	5.1±4.21	42.1±3.11	35.6±3.92	22.4±2.99		
					100.00	1.3±2.03
Dried (set 2)						
500	60.1±2.08	98.8±0.50	98.3±0.96	86.8±4.35		
250	38.0±4.97	84.0±3.16	77.5±4.20	59.0±3.16		
125	20.3±3.78	78.5±1.83	68.5±3.11	46.0±2.45		
62.5	11.2±2.99	59.8±5.16	48.9±3.55	28.1±4.78		
31.25	9.3±5.40	47.9±5.23	40.5±6.18	17.6±6.10		

SD = Standard deviation

Table 3. LC_{50} and regression values for egg hatching and larval development of the plant extracts.

Preparation method	Plant extract	LC_{50} on egg hatching (LCL-UCL)	LC_{50} on Larval development (LCL-UCL)
Poultice paste (Set 1)	*C. papaya* leaves	431.32 (386.74-490.73)	335.00 (271.11-440.89)
	C. papaya seeds	49.94 (22.90-76.25)	49.32 (10.68-87.87)
	T. vogelii leaves	96.92 (49.37-167.26)	57.94 (24.15-92.80)
	T. villosa leaves	101.45 (65.98-148.51)	159.09 (83.59-321.35)
Dried (Set 2)	*C. papaya* leaves	417.83 (327.24-584.32)	386.54 (30.18-541.05)
	C. papaya seeds	48.81 (20.11-76.60)	38.36 (12.98-62.36)
	T. vogelii leaves	73.32 (60.61-86.63)	56.07 (16.73-96.80)
	T. villosa leaves	131.78 (110.40-157.90)	138.80 (116.84-165.92)

poultice paste of *C. papaya* leaves on egg hatching and larval development may be attributed to lack of ovicidal or larvicidal action of the metabolites or the alteration of these compounds by heating. Marie-Magdeleine et al. (2009) suggested that heating could potentially denature bioactive molecules, thereby influencing the anthelmintic activity of aqueous extracts of *Cucurbita moschata*. The study also shows that both the poultice paste extracts and the dried extracts of the plants evaluated showed a dose dependent egg hatching and larval development inhibition at tested concentrations. The probable reasons for the observed minor differences between the poultice paste extracts and dried extracts could be due to similarity of the solubility and the bioactive active constituents. Extracts from *C. papaya* seeds and *T. vogelii* leaves showed dose-dependent inhibition at lower concentration compared to other extracts.

In vivo studies have showed the potency of crude aqueous extract of *C. papaya* seeds against helminthes in sheep (Hounzangbe-Adote et al., 2001; Ameen et al., 2010) and goats (Fajimi et al., 2005) and poultry (Ameen, 2012). Incorporation of *C. papaya* leaves into goat feed resulted to increased feed intake and decrease egg per gram (EPG) in the feaces *in vivo* as well as *in vitro* (Daryatmo et al., 2010). Previous *in vitro* studies have showed anthelmintic effect of ethanolic extract of *C. papaya* seeds (Hounzangbe-Adote et al., 2005). Other studies on non-ruminants have also indicated potential anthelmintic effects of *C. papaya* latex and seeds on helminthes in mice, rats, pigs and poultry (Satrija et al., 1994, 1995; Sapaat et al., 2012; Bi and Goyal, 2012). The anthelmintic activities of *C. papaya* seeds extracts are associated with the presence Benzyl isothiocyanate (Kermanshai et al., 2001). Toxicity studies show that *C. papaya* seeds and leaves are considered safe for livestock and human consumption due to their low contents of oxalate and alkaloids compared to other commonly consumed food products (Adeniyi et al., 2009; Halim et al., 2011).

Crude aqueos extract of *T. vogelii* have shown significant activity against *Ascaridia galli* in indigenous chicken both *in-vitro* and *in-vivo* (Siamba et al., 2007). The methanolic leaf extracts of *T. vogelii* have also shown anthelminthic activity against *Nippostrongylus braziliensis* (Edeki, 1997). There is no scientific evidence of the *in vitro* or *in vivo* anthelmintic activity of *T. vogelii* or *T. villosa* extract in ruminants. However, their anthelmintic effect of these plants could be attributed to the presence of alkaloids, tannins, rotenoids and flavonoids constituents of the leaves (Marston et al., 1984; Ekpendu et al., 1998; Madhusudhana et al., 2010; Ahmad and Khan, 2013). Larvicidal and ovicidal effects of plants with these compounds against gastrointestinal nematodes have been reported in previous studies (Lateef et al., 2003; Siamba et al., 2007).

The extraction of plants in *in vitro* condition is not always comparable to those *in vivo* and as a result the outcome of the two assays can differ (Athanasiadou et al., 2001). *In vitro* tests only provide means for rapid screening for potential anthelmintic activities of plant extracts. The results therefore remain indicative and have to be confirmed through *in vivo* studies with experimental nematode infections in target host species. The potential of the plant aqueous extracts in this study in inhibit egg hatch and larval development may provide an alternative low-cost method for helminthes control, since the plants are available all-year round in Kenya.

Conclusion

Based on the result of this study, it can be concluded that *T. vogelii, T. villosa, C. papaya* leaves and seeds in form of crude aqueous extracts have anthelmintic activity in vitro against gastrointestinal nematodes of sheep. Based on the LC_{50}, the most potent decoction was that of *C. papaya* seeds for both egg inhibition and larval development inhibition. These studies suggest that these plant extracts could form an alternative to commercially available chemical anthelmintics drugs. In view of these findings, further research may be carried out for phytochemical screening and toxicity in order to exploit and verify the use of these plants as crude anthemintic agents. There is need to develop standardized methods for preparations for plants with good anthelmintic activity and formulate best alternative herbal preparation to replace or compliment the chemical anthelmintic drugs currently in use.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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