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In-Vitro Anthelminthic Effects of *Vernonia amygdalina* Leaf Ethanolic and Aqueous Extracts on Earthworms (*Pheritima posthuma*)

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Abstract
Anthelmintic resistance to commonly used chemotherapeutic drugs is on the increase and alternatives are been explored, and medicinal plants have shown promised as probable alternatives to orthodox drugs. This study was conducted to investigate the *in-vitro* anthelminthic effects of crude ethanolic and aqueous extracts of *Vernonia amygdalina* leaf on earthworms (*Pheritima posthuma*). The leaves were collected from the University of Maiduguri campus, air dried, ground into powder using pestle and mortar and extracted. Four graded concentrations (1.25, 2.5, 5.0 and 10.0 mg/mL) of the crude extract for each solvent were prepared and evaluated for the *in-vitro* anthelmintic effects. The number of dead and alive earth worms were counted and recorded after 60 minutes and repeated after 120 minutes. The results indicated that there was an increasing mortality with increasing concentration of the ethanolic extract with regards to percentage dead in number of earthworms exposed for 60 minutes with 15% at 1.25mg/mL and 95% at 10mg/mL with a mean±SD of 0.75±0.5 and 4.75±0.5 respectively(p<0.05). After 120 minutes post immersion, the mortality pattern fn the earthworms followed a similar trend with 35% at 1.25mg/mL and 100% at 10mg/mL with a mean±SD of 1.75±0.5 and 5.0±00 respectively (p<0.05). Similarly, the use of the aqueous extract exhibited similar trend. Anthelmintic activity was higher in the exposed earthworms after 120 minutes compared with 60 minutes post immersion across the different treatment groups and both extract concentrations of 10 mg/mL had a 100% death similar to the positive control (Albendazole, 40 mg/mL). In conclusion, anthelmintic activity of the ethanolic and aqueous fractions of *V. amygdalina* leaf was best observed after 120 minutes of exposure, with increasing activity as the concentration increases with peak action at 10.0 mg/mL.

Keywords: *In-vitro*, Anthelmintic Effects, *Vernonia amygdalina* Leaf, Ethanolic, Aqueous, Extracts, Earthworms

Introduction

Gastrointestinal nematodes are one of the leading causes of morbidity and mortality among small ruminants generating significant economic losses for the livestock industry (Kumarasingha *et al*., 2016). Different anthelmintic drugs are available as control measures for these parasites, but their inappropriate use has resulted in multiple parasite resistance which has led to the loss of their effectiveness (Sánchez *et al*., 2019), together with the possibility of creating ecological imbalances and causing the presence of drug residues in meat for human consumption (Moreno and Lanusse, 2017). Because of the development of resistance in the nematode population, traditional medicinal plants use as an alternative control strategy has increased. African bitter leaf, *Vernonia amygdalina* is a popular vegetable in West and Central Africa (Abosi and Raseroka, 2003), and belongs to the *Asteraceae* family (Tekou *et al*., 2018). Primates like chimpanzees and gorillas in the wild have been suggested to use *V. amygdalina* for self-parasitization (Jisaka et al., 1992, Huffman, 2001). Decoctions of this plant have been used for de-ticking in cattle (Regassa, 2000; Muhimuzi et al., 2019). Furthermore, extracts of V. amygdalina has shown some anti-trypanosomal activity against *Trypanosoma brucei brucei* (Igweh and Onabanjo, 1989), *Leishmania donovani* (Sahpaz et al., 1994) and anthelminthic activity (Ademola and Eloff, 2011). The observed pharmacological activity observed for this plant has been attributed to the

presence of bioactive compounds including sesquiterpene lactones Vernodalin, Vernolide, Hydroxyvernolide, Vernomydin and Vernodal (Alawa et al., 2003) This study was conducted to investigate the *in-vitro* anthelmintic effects of crude ethanolic and aqueous extracts of *Vernonia amygdalina* leaf on earthworms (*Pheritima posthuma*) compared with Albendazole (Albavet®).

Materials and Methods

Plant Collection and Identification

Fresh leaves of *Vernonia amygdalina* were collected at the University of Maiduguri. The specimen was identified and authenticated at the herbarium by a botanist from the Department of Biological Sciences, University of Maiduguri. The leaves were air dried in the Veterinary Parasitology and Entomology Laboratory University of Maiduguri to prevent solar leaching for one week and were ground with pestle and mortar to obtain 1000 g of the leaf powder.

Plant Processing and Extraction

Four hundred (400) grams of the pulverized leaf was extracted in three liters of ethanol using an Ace Soxhlet Extractor 6730 and condenser 6740 (Quick fit, England) at 60^0C for 10 hours. The same procedure was repeated for the aqueous extraction. The extract was concentrated to remove water and was stored at 4^oC for use.

Collection of Earthworms

The earthworm (*Pheritima posthuma)* about 2.0 to 6.5mm in length was used for the study and was collected from the water-logged area in the Agricultural farm of the University of Maiduguri. After collection from the moist soil, the earthworms were washed with water to remove all debri. *Pheritima posthuma* was used due to the anatomical and physiological resemblance with parasitic gastrointestinal nematodes in humans and animals (Nirmal *et al*., 2007).

Preparation and Dilution of Extract

Four different concentrations, 1.25 mg/mL, 2.50 mg/mL, 5.0 mg/mL, and 10.0 mg/mL of extracts of *V. amygdalina* leaf were used for the *in vitro* assay. For each concentration, four (4) Petri dishes of five worms were used for the assay. In total, 20 earthworms were utilized for each treatment group.

Experimental Design

The anthelmintic activity was performed using standard protocol as described by Ajaiyeoba *et al*.,(2001). Earthworms (*Pheritima posthuma)* were counted (5) in number and placed in each petri dish. Four different concentrations (1.25, 2.50, 5.0 and10.0 mg/mL) were used for the *in vitro* assay. After immersion for 60 and 120 minutes, the earthworms were observed for signs of paralysis and death. Death was said to occur when the worm was shaken vigorously and neither moved nor responded to stimuli and color change of the earthworm to pale white. The number of death and alive were recorded. Also, the positive (albendazole®) and negative (physiological saline solution) control experiments were carried out in four replicates.

Statistical Analysis

All analyses were carried out in replicates and data collected were expressed as mean ± Standard deviation (SD). Analysis of variance (ANOVA) was used to analyze the extent of variation among groups. P values of < 0.05 were regarded as significant.

Results

The results of the anthelmintic activity of ethanolic extract of *V. amygdalina* leaf on earthworms exposed to graded concentrations are presented in Table 1. Graded dose response was observed across the different treatment groups for the immersed earthworm. In earthworms immersed for 60 minutes, the number of dead increased in a graded dose manner from 3 (15.0%) to 19 (95.0%). After 120 minutes post immersion, the mortality pattern on the earthworms followed a similar trend ($p<0.05$). Anthelmintic activity was higher in the immersed earthworms after 120 minutes compared to 60 minutes post immersion across the different treatment groups and extract concentrations of 10 mg/mL had a 100% death similar to the positive control (Albendazole, 40 mg/mL).

Table 1: *In vitro* effects of different concentrations of ethanolic extract of *V. amygdalina* leaf on earthworms

Treatment Groups/ Extracts Concentration (mg/mL)	60 Minutes Post Immersion /Exposure				120 Minutes Post Immersion/Exposure			
	No. (%) of Alive	Mean±S.D of Alive (range)	No. (%) of Dead	Mean±S.D of Dead (range)	No. (%) of Alive	Mean±S.D of Alive (range)	No. (%) of Dead	Mean±S.D of Dead (range)
1.25	17 (85.0)	4.25±0.5^{a} (4 - 5)	3 (15.0)	0.75±0.5^{a} (0 - 1)	13 (65.0)	1.75±0.5^{a} (3 – 4)	7 (35.0)	1.75±0.5^{a} (1 – 2)
2.5	10 (50.0)	2.50±0.58^{a} (2 - 3)	10 (50.0)	2.50±0.58^{a} (2 - 3)	7 (35.0)	3.25±1.5^{a} (0 – 3)	13 (65.0)	3.25±1.5^{a} (2 – 5)
5.0	4 (20.0)	1.0±0.82^{a} (0 - 2)	16 (80.0)	4.0±0.82^{a} (3 - 5)	3 (15)	4.25±0.95^{a} (0 – 1)	17 (85.0)	4.25±0.95^{a} (3 – 5)
10.0	1 (5.0)	0.25±0.5^{a}	19 (95.0)	4.75±0.5^{b} (4 – 5)	0 (0)	5±0^{b}	20 (100.0)	5±0^{b}
Albendazole 40 mg/mL (Positive control)	0 (0)	0±0^{bc}	20 (100.)	5±0^{b}	0 (0)	0±0^{bc}	20 (100.0)	5±0^{b}
Normal Saline (Negative control)	20 (100)	5±0^{a}	0 (0)	0±0^{a}	20 (100.0)	5±0^{d}	0 (0)	0 ± 00

Mean ± SD values within columns with different superscripts are significantly (p<0.05) N = 20 (Total number of earthworms immersed to each extract concentration)

The results for the anthelmintic activity of aqueous extract of *Vernonia amygdalina* leaf on earthworms exposed to graded concentrations are presented in Table 2. Graded dose response was observed across the different treatment group for the immersed earthworm. Earthworms immersed for 60 minutes, had number dead increased in a graded dose manner from 20.0% to 55% following treatment. After 120 minutes post immersion, the mortality pattern on the earthworms followed a similar trend. anthelmintic activity was higher in the immersed earthworms after 120 minutes compared with 60 minutes post immersion across the different treatment groups and extract concentrations of 10 mg/mL had a 100% death similar to the positive control (Albendazole, 40 mg/mL).

Table 2: *In vitro* effects of different concentrations of aqueous extract of *Vernonia amygdalina* leaf on earthworms

Treatment Groups/ Extracts Concentration (mg/mL)	60 Minutes Post Immersion /Exposure				120 Minutes Post Immersion/Exposure			
	No. (%) of Alive	(Mean±S.D) of Alive (range)	No. (%) of Dead	(Mean±S.D) of Dead (range)	No. (%) of Alive	(Mean±S.D) of Alive (range)	No. (%) of Dead	(Mean±S.D) of Dead (range)
1.25	16 (80.0)	4.0±0.82^{a} (3 - 5)	4 (20.0)	1.0±0.82^{a} (0 - 2)	15 (75.0)	3.75±0.96^{a} (3 – 5)	5 (25.0)	1.25±0.95^{a} (0 – 2)
2.5	12 (60.0)	3.0±0.0^{a} (3 - 3)	8 (40.0)	2.0±0.0^{a} (0 - 2)	11 (55.0)	2.75±0.5^{a} (2 – 3)	9 (45.0)	2.25±0.5^{a} (2 – 3)
5.0	7 (35.0)	1.75±0.5^{a} (1 - 2)	13 (65.0)	3.25±0.5^{a} (3 - 4)	6 (30)	1.50±0.58^{a} (1 – 2)	14 (70.0)	3.5±0.58^{a} (3 – 4)
10.0	9 (45.0)	2.25±0.5^{bc}	11 (55.0)	2.75±0.5^{b}	0 (0)	0±0^{bc}	20 (100.0)	5±0^{b}
Albendazole 40 mg/mL (Positive control)	0 0)	0±0^{bc}	20 (100.0)	5±0^{b}	0 (0)	0±0^{bc}	20 (100.0)	5±0^{b}
Normal Saline (Negative control)	20 (100.0)	5±0^{a}	0 (0)	0±0^{a}	20 (100.0)	5±0^{d}	0 (0)	0±0

Mean ± SD values within columns with different superscripts are significantly (p< 0.05) N = 20 (Total number of earthworms immersed to each extract concentration)

Discussion

The problem of synthetic drugs and anthelmintic resistance in livestock requires an in look into alternative treatments using natural plant products. In this study the ethanolic and aqueous extracts of *V. amygdalina* leaf displayed a high *in-vitro* lethal activity against earthworms and could be a folkloric evidence for agro-pastoral farmers giving credence to the use of *V. amygdalina* as an antihelmintic in livestock (Wasswa and Olila, 2006; Nalule *et al.*, 2011). This *in-vitro* activity in this study could be attributed to the presence of phytochemicals such as alkaloids, tannins, glycosides, triterpenoids, flavonoids, anthracenoides, anthracyanins, coumarin derivatives and saponins (Makut *et al.*, 2008;Danquah *et al*., 2012; Nalule *et al*., 2013), either singly or in combination to cause inhibition and paralysis and death of the worms. Tannins in plant extracts have anthelmintic activity on their own attributed to physical astringent action on helminthes (Molan *et al*., 2000). Furthermore, previous studies have shown that the ethanolic and water extracts of *V. amygdalina* inhibited *Ascaris suum* motility in a dose dependent response by paralyzing them or causing their death (Nalule *et al*., 2013). The two fractions of the extract were effective, both exhibiting good anthelmintic activity. However, on comparative basis, the ethanolic extract was better in inhibiting motility and death of the earthworms than the water crude extracts of *V. amygdalina*. This could probably be related to the different chemical components extracted in the different solvents due to their polarity and their biological effects on the earthworm. The study showed that efficacy of extracts increased with increasing concentration of extract. The concentration dependent activity of the extracts suggests that the increase in concentration of plant extract is followed by a supplementary input of different active compounds. The mechanism of the anthelmintic action is yet to be determined but we speculate that the extract paralyzed the earthworms resulting in death. Most natural products exhibit their antiparasitic activity through the interference with the redox balance of the parasites, by either acting on the respiratory chain or the cellular defenses against oxidative stress. This is because natural products possess structures capable of generating radicals that may cause peroxidative damage to the pathogen. In conclusion, anthelmintic activity of the ethanolic and aqueous fractions of leaf of *V. amygdalina* was best observed after 120 minutes of exposure, with increasing activity as the concentration increases with peak action at 10.0 mg/mL**.**

Conclusion

The ethanolic and aqueous extracts of *V. amygdalina* showed promising effects as a potential anthelminthic using the earthworm model. The anthelminthic activity was most pronounced after 120 minutes of exposure in a dose-dependent manner. We speculate that the bioactive compounds present in the extract could be responsible for the observed effects. However, further studies are required to determine the phytochemical, elemental and antioxidant activities of the plant extract and the probable mechanism of action of the plant extracts.

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