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THE EFFICACY OF *Vitex doniana* AND *Boswellia dalzielii* AGAINST EGG HATCH OF *Caenorhabditis elegans*

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ABSTRACT
This study evaluates the efficacy of aqueous and methanolic extracts of V. doniana and B. dalzielii in vitro against the egg hatch of C. elegans DA1316 (ivermectin resistant strain) and C. elegans Bristol N2 (wild type). The eggs and larvae were incubated in aqueous and methanolic extracts of the stem bark of V. doniana and B. dalzielii with the serial concentrations of (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml). The extracts of V. doniana was effective against the egg hatch of C. elegans DA1316 as only 16.2% and 7.7% egg hatch were recorded in the aqueous and methanolic extracts respectively at 2.0 mg/ml. Boswellia dalzielii was ineffective as up to 46.2% and 30.9% egg hatch was recorded in the aqueous and methanolic extracts respectively against C. elegans DA1316. The effects of the aqueous and methanol extracts of V. doniana on the egg hatch of C. elegans Bristol N2 was statistically the same with that of C. elegans DA1316 (P > 0.05), likewise that of aqueous and methanolic extracts of B. dalzielii. A significant difference was established in the percentage ovicidal and larvicidal efficacy between aqueous and methanol extracts (P < 0.05) against both strains of C. elegans.
Key words: aqueous; effective; extracts; inhibition; ovicidal

INTRODUCTION

One of the major global pathological threats to the management of small ruminants is gastrointestinal nematode's infections. Nematodes are increasingly developing resistance to synthetic drugs because of long-term repeated exposure. Also, development of multiple resistances to commercial anthelmintic by the parasites is on the increase (Hoste *et al.,* 2015; Jackson *et al.,* 2012). Serious economic and production loss are often encountered due to gastrointestinal nematodes infections. The economic loss is measured in terms of resources wasted on treatment and research. Production lost referred to lose in the quantity and quality of the products obtained from the animals such as hide and skin, hair, meat and milk (Besier *et al.,* 2015). Researchers have explored plant as an alternative to conventional drugs considering the development of resistance to modern chemotherapy, the side effect of synthetic drugs, residual effects of the drugs in the food and environment coupled with the cost of the synthetic drugs (Nery*et al.,* 2009).

In vitro test is the first line of action in screening of plants for anthelmintics. However, high cost of *in vitro* screening, ethical constrain and difficulty in obtaining certain suitable stage of life cycle of a parasitic organism is among the challenges facing *in vitro* screening of plants for anthelmintics in the drugs development process. These challenges have been overcome with the discovery of *C. elegans* as a model in several researches. *Caenorhabditis elegans* is a free-living nematode whose characteristic such as short generation time, easy maintenance, among others give it an advantage above other nematodes in the *in vitro* screening for drugs (Simpkin *et al.,* 1981). *Caenorhabditis elegans* belongs to the same clad (V) with parasitic nematodes such as *Haemonchus contortus, Trichostrongylus colubriformis* and *Ostertagia ostertagi* and they share common anatomical and physiological features. Therefore, it is reasonably assumed that drugs effects on *C. elegans* or any of its developmental stage might be reproduced on these parasitic nematodes (Kumarasingha *et al.,* 2014).

A decoction from the stem bark of *V. doniana* and *B. dalzielii* have been used as dewormers as well as for treating diarrhoea and gastrointestinal disorder by pastoralists in Nigeria. This research aimed at evaluating the efficacy of crude aqueous and methanol extracts of *V. doniana* and *B. dalzielii* against egg hatch of *C. elegans* DA1316 and *C. elegans* Bristol N2 based on the following objectives:

i. To test the efficacy of the extracts on the egg hatch of *C. elegans* DA1316 and *C. elegans* Bristol N2
ii. To compare the ovicidal efficacy of extracts from the stem bark of *V. doniana* against that of *B. dalzielii*
iii. To determine the difference in the ovicidal efficacy of aqueous and methanol extracts.

MATERIALS AND METHODS

Collection of Plant Materials and Extraction

Stem barks of Plants were collected in the wild forest of Azare, in Katagum Local Government Area of Bauchi State, Nigeria. Plants were authenticated at the Biological Science Department, Bauchi State University, Gadau. Voucher specimens of the plants with No. 3105 and 900056 for *V. doniana* and *B. dalzielii* respectively were deposited in the herbarium of the department.

Phytochemical Extraction of Plant Materials

The samples were pulverized into powdered form after shed dried. Total of 50 g of each plant powdered sample was macerated in 250 ml (1:5 w/v) of distilled water for five days at room temperature. The aqueous infusion was filtered through a Whatman number 1 filter paper No1. The filtrate was concentrated and dried in an oven at 45^{o}C. The same method was applied for methanolic extraction where 80% methanol was used. The dry extracts were preserved in labeled sterile specimen vials at 4^{o}C for further use (Lienou *et al.,* 2015).

Phytochemical Tests

Phytochemical test which detected presence of secondary metabolites was carried out on the plant's extracts using several types of reagents such as Dragendorff's reagents (alkaloids), Salkowski's test (terpenoids), froth formation on shaking with water (saponins), extract solution mixed with ferric chloride ($FeCl_3$) solution (phenols)extract solution mixed with 10% potassium hydroxide (tannins). Acetic anhydride $(CH_3CO)_2O$) mixed with concentrated sulfuric acid (H_2HSO_4) (steroids) (Tadesse *et al.,* 2015).

Total phenolic Content (TPC) and Total Tannin Content (TTC)

The total phenolic content was carried out according to the method of Orak, (2007) using Folin-Ciocalteau reagents. Gallic acid serial concentrations of (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 mg/ml) were prepared in 50 % (w/v) methanol as standard. A calibration curve of absorbance values against the varying concentrations of the Gallic acid standard was plotted and the regression equation (y = ax + b) of the curve was obtained using Microsoft Excel software 2016. This equation was used to calculate the phenolic content of the samples expressed as Gallic Acid Equivalence in mg (GAE/mg) of the samples. For total tannic content quantification, Folin-Denis spectrometric method described by Oliveira *et al,* (2009) was used. However Tannic acid concentrations of (0, 0.5, 1, 1.5, 2, 2.5 mg/ml) were used as standard. Instead of Folin-Ciocalteau reagent, Folin-Denis reagent was used. The tannin content of each extract was expressed as Tannic Acid Equivalent (TAE/ mg/ml).

Collection and preparation of eggs

Eggs and gravid adults were collected by addition of about 7 ml of M9 solution (3 g KH_2PO_4, 6 g Na_2HPO_2, 5 g NaCl and 1ml of 1M $MgSO_4$ + 1 liter of distilled water) from 3 old plates of the require strain of *C. elegans*. The mixture was transferred to a 15ml centrifuge tube and centrifuged for 2 minutes at 1500 rpm and the clear supernatant was discarded. About 5 ml of fresh bleaching solution (mixture of 5M NaOH and 5 % solution of sodium hypochlorite in the ratio of 1:2) was added to the content and shaken vigorously with occasional Vortexing for 3 minutes until most of the adult worms were dissolved. Approximately 9 ml of the M9 solution was added to stop the bleaching process and the content was centrifuged for 2 minutes at 1500 rpm to clean the egg of bleaching solution. The eggs were re-suspended in M9 solution and the concentration was adjusted to contain 100 eggs per 50 µml of the suspension (Radman, *et al.,* 2013).

Egg hatch bioassay

The stock solution of the plant extracts was prepared by first dissolving 200 mg of the required dry extract in 5 ml of 1% Dimethylsulphoxide (DMSO). M9 solution was used for further dilution of the stock solution to give the serial concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml. Ivermectin Solution was prepared by first dissolving dry sample in (DMSO) and subsequently diluted to 0.02 µg/ml using M9 buffer solution. 50 µml of the suspension containing about 100 eggs was added to each of the 24 macro-wells. Up to 1 ml of each of the concentration the required plant extract was applied to the eggs in the macro-wells in triplicates. Ivermectin solution was applied to the eggs in 3 of the macro wells and served as positive control. M9 buffer was also applied to eggs in another set of 3 wells and served as negative control. About 10 µml of 5 µg/ml of amphotericin B was added to each well to inhibit fungal growth in the macro wells.

The set up was incubated for 24 hours at 20°C. The experiment was repeated three times and the number of L1 larvae that hatched was counted at the end of each test and the percentage egg hatch was calculated according to the formula of Molan (2014) as follows:

$$EHA\% = \frac{Numeber\ of\ larvae\ dead\ or\ alive}{Number\ of\ eggs\ incubated} \text{ x100}$$

Where EHA% = percentage egg hatch

Statistical analysis

The means percentage egg hatch activity of different concentration with the control was compared using one - way ANOVA. The post hoc statistical significance used was least square difference (LSD) and the difference between the means was considered significant at $P < 0.05$. Computation of extract concentration required to inhibit 50% egg hatch (IC_{50}) was carried out using probit analysis.

RESULTS AND DISCUSSION

The methanolic extract of each of the plant revealed more types of metabolites than the aqueous extract (Table 1), in line with the findings of Badar *et al.* (2011). Also, methanolic extract contained higher quantities of phenolic and tannins content than the aqueous extract of both plants ($P < 0.01$) as shown in Table 2 below. A higher quantity of tannic compounds in the methanol extracts than in the aqueous extract of several plants was also reported by Neffati *et al.* (2017). According to Iloki-Assanga *et al.* (2015), water as a polar solvent can only extract a polar compound whereas methanol which is a polar as well as none polar solvent, extract both polar and none polar compounds thereby extracting more varieties and quantity of metabolites hence resulting in more varieties and quantities of metabolites in methanol extracts than in aqueous extracts. In addition, the non-polar characteristic of methanol enables it to dissolve the non-polar plant cell walls of the plants to release more types and quantities of secondary metabolites (Tiwari *et al.,* 2011).

The results revealed low values of IC_{50} and low percentage of egg hatch as the concentration increased up to 2.0 mg/ml. For instance, aqueous extract of *V. doniana* which was moderately effective exhibited the IC_{50} of 1.025 mg/ml and only 16.2% egg hatch against *C. elegans* DA1316 at 2.0 mg/ml whereas the methanol extract which was effective exhibited the IC_{50} of 0.66 mg/ml and 7.70% eggs hatch at 2.0 mg/ml (Table 3 and 4). Similarly, The IC_{50} of 0.932 mg/ml and 15.5% egg hatch was recorded in the aqueous extract of *V. doniana* at 2.0 mg/ml against *C. elegans* Bristol N2 compared to the IC_{50} of 0.762 mg/ml and 6.5% egg hatch recorded in methanol extract (Table 3 and 4). *B. dalzielii* was ineffective as the aqueous extract recorded the IC_{50} of 2.011 mg/ml and 46.2% egg hatch against *C. elegans* DA1316 at 2.0 mg/ml whereas methanol extract recorded the IC_{50} of 1.810 mg/ml and 30.9% egg hatch. Furthermore, the aqueous extract of *B. dalzielii* recorded the IC_{50} of 1.891 mg/ml and 44.2% egg hatch against *C. elegans* Bristol N2. On the other hand, the IC_{50} of 1.642 mg/ml and 28.1% egg hatch was recorded by methanol extract against the egg hatch of *C. elegans* Bristol N2.

Currently, there is a scarce scientific experimental report on the anthelmintic activity of *V. doniana and B. dalzielii*. However aqueous and ethanolic extract of *V. doniana* was reported to be antimicrobial, anti diarrhoea and the infusion from stem bark was used for the treatment of gastrointestinal infection (Kubmarawa *et al.,* 2007). Methanolic extracts of stem bark of *B. dalzielii* was reported to be anti-trypanosomal (Atawodi *et al.,* 2011).

Generally, the performance of the plant extracts was concentration dependent as reported in a similar research by (Kanojiya *et al.,*2015; Ndjonka *et al.*, 2014). From the above results, *V. doniana* extract was more potent in the inhibition of egg hatch than extracts of *B. dalzielii*. This could be attributed to more varieties of secondary metabolites and higher phenolic and tannin contents recorded in the extracts of *V. doniana* than the extracts of *B. dalzielii* ($P < 0.05$). Badar *et al*. (2011) suggested that the potency of an extract of stem bark of *Acacia nilotica* more than the leaves against the Motility of *H. contortus* was atttributed to the disparity in the types/quantity of secondary metabolites in the extract from stem bark compared to leaves. The methanol extract of each of the plant was more ovicidal than the aqueous extract. This could also be due to higher phenolic and tannin content in the methanol extract compared to that of aqueous extract ($P < 0.05$). Futher more, the enzyme polyphenol oxidase degrades polyphenol in aqueous extracts thereby reducing the potency of the extract whereas the enzyme is inactive in methanol extracts (Tiwari *et al.,* 2011). Other reasons for the higher performance of methanol extracts than the aqueous extract could be due to easy trans-membrane absorption of methanol extract than aqueous extract as explain by Getachew *et al.* (2012). Ivermectin was more effective than the plant extracts ($P < 0.05$) against egg hatch of *C. elegans* Bristol N2.

This might be attributed to the presence of impurities in the crude extracts of the plants which might have interfered with the potency of the plant, unlike ivermectin which contains only pure compound of high efficacy. Also, ivermectin might exhibits a different mode of action against the eggs and the larvae which may result in the variation in the potency of the extracts and ivermectin in line with the assumption of Al-Rofaai *et al.* (2012).

Table (1). Secondary metabolites present in methanol and aqueous extracts of *V. donianaand B. dalzielii*

Aqueous methanol														
	alk	sap	tan	ter	ste	fla	phe	alk	sap	tan	ter	ste	fla	phe
V. doniana	-	+	+	+	-	+	+	+	+	+	+	+	+	+
B. dalzielii	+	-	+	-	+	-	+	-	+	+	+	-	+	+

Key: alk = Alkaloids, sap = saponins, tan = tannins, ter = terpenoids, ste = steroids, fla = flavonoids, phe; phenols, + = presence of metabolite, - = absence of metabolite.

Table (2): Total tannins content TAE/mg ± Standard deviation of extracts of *V. doniana* and *B. dalzielii*

Plant	**Phenolic contents (GAE/mg ± SD)**		**Tannins content (TAE/mg± SD)**	
	Aqueous extract	**Methanol extract**	**Aqueous extract**	**Methanol extract**
V. doniana	326.74 ± 2.31	462.91 ± 2.5	3.99 ± I.9	5.70 ± I.2
B. dalzielii	16.2 ± 0.10	21.53 ± 0.131	0.33 ± 0.03	0.7 ± 0.075

Table (3): IC50 of Aqueous and Methanol extracts of *V. doniana* and *B. dalzielii* Against Egg hatch of *C. elegans* DA1316 and *C. elegans* Bristol N2

Plant/Extract	**Egg hatch DA1316**	**Bristol N2**
V. doniana	IC_{50} mg/ml	IC_{50} mg/ml
Aqueous	1.025	0.932
Methanol	0.66	0.762
B. dalzielii		
Aqueous	1.891	2.011
Methanol	1.642	1.810

Data was based on percentage ± standard error of 3 independent experiments.

Table (4).Percentage ± Standard Error Ovicidal Efficacy of Aqueous and Methanol extract of *V. doniana* against *C. elegans* DA1316 and *C. elegans* Bristol N2

Conc. mg/ml	***C. elegans* DA1316 Aqueous extract**	**Methanol extract**	***C. elegans* Bristol N2 Aqueous extract**	**Methanol extract**
0.2	92.0 ± 0.42	86.1 ± 0.76	88.6 ± 0.68	83.3 ±0.34
0.4	83.0 ± 0.72	70.8 ± 0.78	74.9 ± 0.67	67.1 ± 0.52
0.6	68.7 ± 0.74	63.9 ± 0.44	65.7 ± 0.74	62.1 ± 0.41
0.8	56.4 ± 0.67	48.3 ± 0.61	54.4 ± 0.69	47.4 ± 0.61
1.0	40.6 ± 0.72	28.3 ± 0.58	34.3 ± 0.55	30.0 ± 0.78
2.0	16.2 ± 0.59	7.70 ± 0.56	15.5 ± 0.61	6.50 ± 0.61
Ivermectin	94.3 ± 0.55	95.2 ± 0.58	5.20 ± 0.52	6.40 ± 0.55
Negative. control	98.0 ± 0.30	97.2 ± 0.61	96.3 ± 0.33	96.7 ± 0.33

Data was based on percentage ± standard error of 3 independent experiments.

Table 5: Percentage ± Standard error ovicidal efficacy of Aqueous and Methanol extract of *B. dalzielii* against *C. elegans* DA1316 and *C. elegans* Bristol N2

Conc. mg/ml	*C. elegans* DA1316 Aqueous extract	Methanol extract	*C. elegans* Bristol N2 Aqueous extract	Methanol extract
0.2	96.7 ± 0.68	84.4 ± 0.69	99.8 ± 0.66	90.0 ± 0.78
0.4	94.3 ± 0.47	78.8 ± 0.68	96.6 ± 0.52	83.0 ± 0.38
0.6	89.1 ± 0.73	75.4 ± 0.64	92.2 ± 0.55	75.9 ± 0.75
0.8	84.2 ± 0.58	68.9 ± 0.64	88.2 ± 0.55	68.7 ± 0.76
1.0	75.0 ± 0.43	59.7 ± 0.67	81.3 ± 0.59	62.5 ± 0.70
2.0	46.2 ± 0.52	30.9 ± 0.52	44.2 ± 0.46	28.1. ± 0.63
Ivermectin	96.2 ± 0.49	97.1 ± 0.55	4.99 ± 0.64	5.54 ± 0.58
Negative control	96.5 ± 0.35	96.8 ± 0.93	98.2 ± 0.61	96.3 ± 0.88

Data was based on percentage ± standard error of 3 independent experiments.

CONCLUSION

Methanolic and aqueous extract of *V. doniana* were effective against egg hatch of *C. elegans* DA1316. Methanol was more efficient than aqueous extract. *B. dalzielii* was ineffective against egg hatch of *C. elegans* DA1316. Ivermectin was ineffective against the egg hatch of *C. elegans* DA1316. *V. doniana* can be use as a natural source of a lead compound to produce anthelmintic.

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Conflict of interest

Authors declare that there were no any conflicts of interest in doing this research.

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