

Research Article

In vitro Anthelmintics Efficacy and its Utilization Practices Studies against *Haemonchus contortus* and *Trichostrongylus colubriformis* in Ada'a District, Oromia Region, Ethiopia

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- Ivermectin
- Levamisole
- *Trichostrongylus colubriformis*

Abstract

Background: The worldwide increased difficulty of anthelmintic resistance (AR), calls for an enhanced and standardized implementation of early detection of AR. This study aims to determine efficacy of anthelmintic drugs against *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Ada'a district in Ethiopia.

Methods: Structured questionnaire and *In vitro* studies were conducted to evaluate the efficacy of different anthelmintic brands in serial dilutions of 1.0, 0.5, 0.25, 0.125, and 0.0625 µg/ml. *In vitro* egg hatch test (EHT) assay, One hundred (100) eggs per well of *Haemonchus contortus* and *Trichostrongylus colubriformis* were incubated with the drugs at 27°C for 48 hours. In the Larval motility test, 50 infective larvae (L3) of parasites mixed with the drug of choice and incubated at 25°C for 24hrs after which motile and non-motile worms were counted. In the adult motility test, 15 adult *H. contortus* per well were mixed with the test drugs and incubated at 37°C for 10 hours while each test performed in triplicates.

Results: The tested anthelmintic brands significantly showed concentration-dependent responses on eggs, larvae, and adult parasites. The Effective Concentration (EC50) result of EHA was found to be significant only for China (CN) brand of albendazole against both *Trichostrongylus colubriformis* (0.087 µg) and *Haemonchus contortus* eggs (0.017 µg); whereas the EC50 test result on levamisole, brands indicated that East Africa (EA) brands (0.057 µg) were found to be significantly effective against infective stages (L3) of *Trichostrongylus colubriformis*; whereas both brands of East Africa (EA) and India (IN) were significantly effective against both *Haemonchus contortus* L3 (0.058 µg) and L3 of *Trichostrongylus colubriformis* (0.048 µg). The EC50 of ivermectin was showed a significant difference against both parasites isolates respectively within China brands for L3 of *H. contortus* and *T. colubriformis* and Indian brands for L3 of *T. colubriformis*. However, the EC50 done for tetramisole brands were suggested a lower efficacy of IN brands (5.576 µg) than CN brands.

Conclusions: China origins of the tested albendazole (ABZ), ivermectin (IVM), and tetramisole (TTM) brands were found to be more effective against eggs, infective stages (L3), and adults of the parasites concerned: On the other hand, LEV from East Africa Company is proven effective against L3 of both parasites whereas LEV and IVM from Indian origin are effective only against L3 of one species at acceptable concentration.

BACKGROUND

Ethiopia is home to large and diverse livestock resources and favorable production environments. Particularly, the vast majority of the rural population's livelihood is partly based on livestock production where about 40% of the cash income earned by farm households and 25% of total domestic meat consumption is derived from small ruminants [1]. Small ruminants represent the most important part of the Ethiopian livestock system, with about 24.2 million sheep and 22.6 million goats reported in the country. In Ethiopia, small ruminants are reared in all agro-climatic zones. The highland area comprises 75% of the sheep and 27% of the goat population, while the lowland pastoral and agro-pastoral areas have 25% of the sheep and 73 % of the goats' population [2]. Small ruminants also have various social and cultural functions that vary among different cultures,

socio-economies, agro-ecologies, and locations in tropical and sub-tropical Africa. Mixed crop-livestock production practice is a common production system of Ethiopia across different agro-ecological conditions which depends on the indigenous breed of a small ruminant with low-level production and productivity. The description of small ruminant production systems in Ethiopia emphasized the contribution of the indigenous breed of small ruminants to the total household revenue [3].

Anthelmintic resistance of gastrointestinal nematode (GIN) parasites constitutes a major problem for livestock health and productivity around the world. In sheep and goats, the situation is the most severe, with an increasing number of farms around the world experiencing resistance to all classes of available anthelmintics [4-7]. Parasitism by gastrointestinal nematodes (GINs) is a major constraint in the production of goats in

tropical countries. They can affect production through weight loss, diarrhea, anemia, reduction in milk and wool production, reproduction changes as well as mortality in the case of heavy infestations [8,9]. A majority of nematodes affecting the intestinal tract of small ruminants belong to the Order Strongylidae, with principal genera being *Haemonchus*, *Teladorsagia*, *Cooperia*, *Trichostrongylus*, *Nematodirus*, *Chabertia*, and *Oesophagostomum* [10]. Compared to other nematodes, *H. contortus* is by far a highly pathogenic parasite of small ruminants, capable of causing acute disease and high mortality in all classes of livestock [11].

Health disorders in all classes of small ruminants represent a factor that greatly affects the economics of sheep and goat production. The most serious problem confronting sheep and goats production worldwide is infection with GIT parasites [12]. Parasitism ranks high among the factors that limit the productivity of small ruminants although its effect is often underestimated. Correspondingly the current levels of contributions of the small ruminants in Ethiopia, either the macro or micro level is below the expected potential [13]. Among major constraints hindering the productivity of sheep and goat in the country, diseases are the principal one; of which endoparasites accounts a wide range of health problems that confront the productivity of small ruminants [14].

The use of anthelmintics has been practiced for a long time and constitutes a considerable share of the costs spent by the country in the control of helminths. Meanwhile, the risk of underdosing and continued use of very limited classes of anthelmintics, irrespective of efficacy status are frequently encountered on many farms [10,15-18]. Also, smuggling and misuse of veterinary drugs involving anthelmintics is a widespread practice in the

country. Some of these drugs, particularly albendazole and tetramisole, have been continuously imported and distributed to every corner of the country under different trade names and by different manufacturers. The extensive and indiscriminate use of the drugs has resulted in the development of resistance [19].

Effective methods that can preserve and maintain the efficacy of anthelmintics, and delay or prevent the emergence of anthelmintic resistance are not practiced in any part of the country [20]. On the other hand, no systematic surveys have been carried out to evaluate the anthelmintic resistance situation in small ruminants, particularly in sheep and goats. The incidences of anthelmintic resistance in small ruminant nematodes have been reported from studies conducted in different parts of the country [5]. This is perhaps an alarming signal for the incoming challenges with worm management in small ruminants in Ethiopia [6,21,22]. This study was therefore aimed at assessing worm control practices in the use and to compare in vitro susceptibility of *H. contortus* and *T. columbriformis* to four brands of anthelmintics commonly available and used in the region to control sheep nematodes in Ada'a district of Central Ethiopia.

MATERIALS AND METHODS

Study Area

The study was conducted in Bishoftu town and its surroundings (Figure 1). The town is located 45 km East of Addis Ababa at 9°N latitude and 4°E longitudes, at an altitude of 1850 m above sea level in the central Oromia Region. The area has an annual rainfall of 86.6-millimeters (mm) of which 84% falls during the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures of the town are

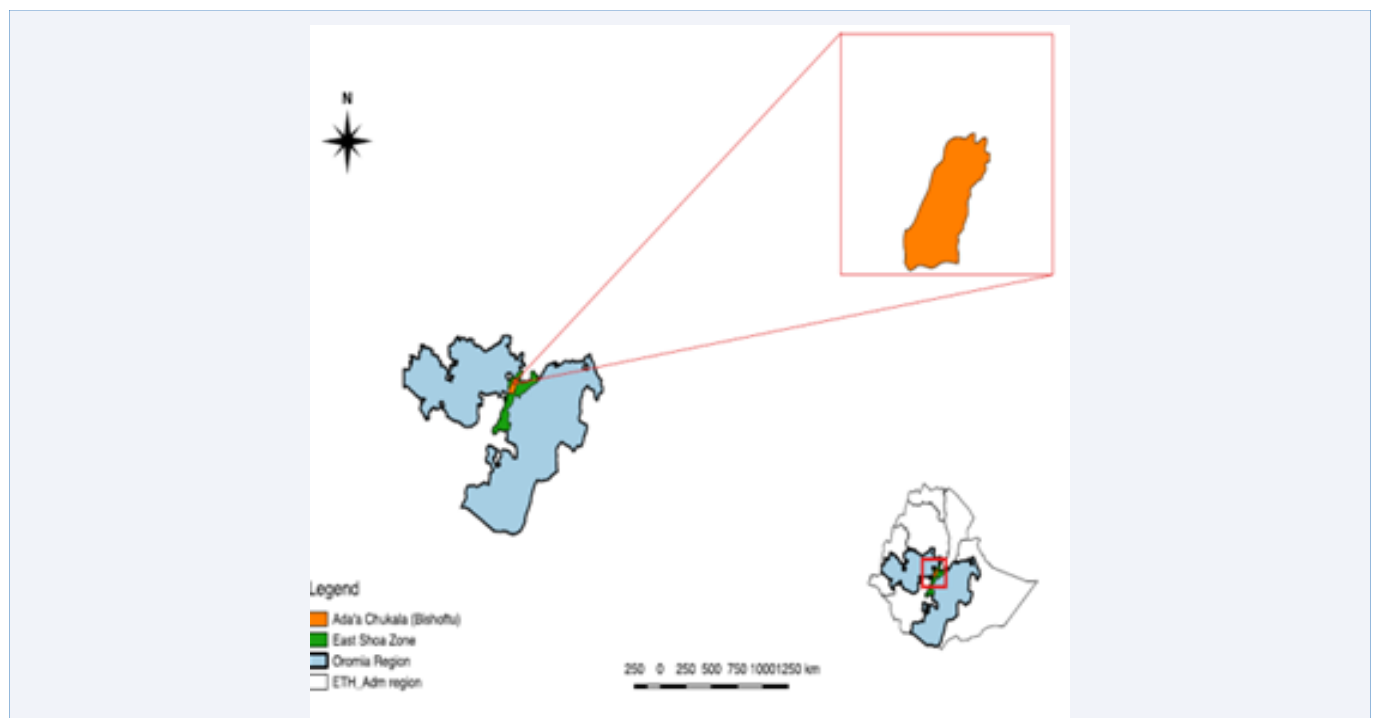


Figure 1 Map of Bishoftu town in East Shoa Zone of Oromia Regional State (Source; Worku and Bedanie, 2019).

26 and 14°C, respectively, with a mean relative humidity level of 61.3%. The mixed crop-livestock production system is the common management system in Bishoftu town (NMSA, 2005). Bishoftu is the capital town of Ada'a district, which is one of the districts in the East Shoa Zone of the Oromia Region and located 47 km southeast of Addis Ababa with a human population of about 95,000 (CSA, 2007.,2009). The district covers an area of 92, 751.33 ha. The average altitude is about 1880 meters above sea level. The average annual rainfall is about 839 mm and the average temperature is 24°C [23].

Questionnaire survey

A questionnaire survey was performed on the 100 sheep and goat owners in the different PAs of Ada'a district. The survey was conducted by way of personal interviews of farmers in a language they can communicate to complete the questionnaire data. After introducing the objectives of the study and obtaining their full consent, one hundred (100) small ruminant owners and fifteen (15) veterinary drug stores and pharmacies were included and interviewed for data collection in the study area according to the previous methods [20,22]. The sample size of the respondents was determined using the formula ($n = 0.25/SE^2$) proposed previously [24], at the standard error (SE) of 0.05 with a 95% confidence interval. Based on this, a hundred (100) small ruminant owners (farmers) having 5 to 15 animals were randomly selected and interviewed according [24].

The questionnaire focused mainly on information on the frequency of use, criteria for selection or choice, main source, and rotation of anthelmintics, who administer the animals, observations on the responses of treatment (efficacy), and educational background of each participant. Only those owners who have reared their sheep and goats were included in the questionnaire survey. Animal traders were not included as they keep the animals for a short duration and hence were not expected to have enough information about their animals according to previously done [25]. To know the exact number of sheep owners to be interviewed, we don't know the total number of households owning livestock in Ada'a districts. Therefore using the worst-case scenario; fifty of (50%) of the respondents giving a "Yes" response to a yes or "No" questions have been expected. Then sample size was calculated according to the earlier formula [24]. Therefore, taking a standard error of 5%, and use the formula which says "Sample Size (N) = 0.25/SE²", one hundred (100) respondents were interviewed to compile the data designed for the questionnaire survey.

Study Animals and parasites

A survey of gastrointestinal parasites was done in different places of the Ada'a district to identify donor sheep with high nematode egg count. Two animals were purchased by negotiation with the owners. These animals were humanely slaughtered to recover adult parasites to get pure *Haemonchus* from the abomasums and pure *Trichostrongylus* from the small intestine. Then, the worms were washed and crushed to liberate eggs. The eggs were then cultured in a plastic jar filled with sterilized cattle feces for 10 days at room temperature according to the previous method [26].

At the end of the ten days, the infective (L3) larvae were harvested [27]. Two new sheep free of helminth parasites and aged 12 and 24 months were purchased from the local market for the propagation of the parasites to be used in vitro studies. They were acclimatized for 2 weeks in a fly-proof animal facility found in the College of Veterinary Medicine and Agriculture. Then, the recovered L3 have drenched to the animals; one species for each animal. The animals were provided with grass hay supplemented with a concentrating facility. All animals were handled according to the guidelines for experimental animal use and management. Following, the establishment of the infections, the daily fecal collection was done for harvesting L3 to be used in larval motility test (LMT) and for recovering eggs to be used in egg hatch assay (EHA). Adult parasites required for the adult motility test were obtained by humanely slaughtering sheep infected by mono-specific parasites after the EHA and LMT were completed according to the protocols recommended by [28-31].

In vitro anthelmintic efficacy studies

Anthelmintics used in this study: The anthelmintics used for the experiment were bought from local retail markets and composed of three drug classes (Table 1). Before, the anthelmintic was purchased; information was gathered on anthelmintic utilization practices in the area and the common drugs available on the local market [32-34]. Then the efficacy of different brands of albendazole, Ivermectin, and Levamisole and Tetramisole were tested using the larval motility (LMT) and egg hatch assay (EHA) methods (Table 1) and interpreted according to the guidelines provided by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) recommendations [33,35,36], for efficacy evaluations of Anthelmintic [33,37-40].

Preparation of serial dilutions of Anthelmintics for in vitro efficacy study

Table 1: Details of the anthelmintic drugs used in vitro experiment.

Drug groups	Generic name	Trade name	Manufacturer
Benzimidazole	Albendazole	Azole	East Africa Pharmaceuticals, Ethiopia
	Albendazole	Albenda	Chengdu Qiankun veterinary Pharmacy
Imidazothiazole	Tetramisole	Tetsole®	East Africa Pharmaceuticals, Ethiopia
	Tetramisole	Ashitetra 600 mg	Ashish Life Science Pvt Ltd, India
	Levamisole	Nizal-QK bolus	Ashish Life Science Pvt Ltd, India
	Levamisole	Zanisole	East Africa Pharmaceuticals, Ethiopia
Macrocyclic Lactones	Ivermectin1%	Tecmectin (1%)	Chengdu Qiankun veterinary Pharmacy
	Ivermectin1%	Ivermic (1%)	Uruguay, Veterinary drug store, Belgium
	Ivermectin1%	Ashiver (5 mg)	Ashish Life Science Pvt Ltd, India brands

For egg hatch assay: The stock solutions of albendazole of Chinese, East Africa, and Indian brands were prepared at concentrations of 1000µg/ml by dissolving 100mg of albendazole in 20 ml of Dimethyl sulfoxide solution and 80 ml of distilled water. The second mother solution of dissolved Albendazole was prepared at concentrations of 500µg/ml by mixing 50 mg of mother solutions with 50 ml of distilled water. The third concentration of Albendazole solution was prepared at concentrations of 250µg/ml by mixing 25 mg of first mother solution with 75 ml of distilled water. The fourth concentration of Albendazole solution was prepared by dissolving 12.5 mg of mother solutions in 87.5 ml of distilled water. The fifth final solution of Albendazole was prepared by dissolving 6.25 mg of Albendazole solutions in 93.75 ml of distilled water. The prepared anthelmintic serial concentrations were then added to the microdilution plates at final dilutions of (1 µg, 0.5 µg, 0.25 µg, 0.125 µg, and 0.0625 µg/ml) to be used directly for the *in vitro* cultivation of isolated parasite eggs according to [17,41].

For Larval motility test: Macrocyclic lactones group of anthelmintic were tested using larval motility test, to evaluate the percentage mortality of infective stage (L3) recovered from *Haemonchus contortus* and *Trichostrongylus. Columbiformes* in sheep. Therefore, the stock solutions of different ivermectin and levamisole brands were prepared at final concentrations of (1 µg, 0.5 µg, 0.25 µg, 0.125 µg, and 0.0625µg/ml) as described in Albendazole serial preparations according to the procedures described by Belew and Kumsa, Eguale [17,41,42].

For adult motility test: tetramisole of Indian and Chinese brands were tested against adult parasites of *Haemonchus. contortus* and each tested concentrations were the same used in albendazole, ivermectin, and levamisole solutions. The stock solutions of tetramisole of Chinese and Indian brands were prepared at concentrations of 1000µg/ml by dissolving 100mg of tetramisole in 20 ml of Dimethyl sulfoxide solution (DMSO) and 80 ml of distilled water. The second mother solution of dissolved tetramisole was prepared at concentrations of 500µg/ml by mixing 50 mg of mother solutions with 50 ml of distilled water. The third concentration of albendazole solution was prepared at concentrations of 250µg/ml by mixing 25 mg of first mother solution with 75 ml of distilled water. The fourth concentration of albendazole solution was prepared by dissolving 12.5 mg of mother solutions in 87.5 ml of distilled water. The fifth final solution of albendazole was prepared by dissolving 6.25 mg of tetramisole solutions in 93.75 ml of distilled water. The prepared anthelmintic serial concentrations were then added to the microdilution plates at final dilutions of (1 µg, 0.5 µg, 0.25 µg, 0.125 µg, and 0.0625 µg/ml) to use directly for the *in vitro* cultivation of isolated parasites eggs according to Belew and Avinash [41,43].

Egg Hatch Assay or Test (EHA/EHT)

In egg hatch assay, the benzimidazole group of anthelmintic was tested to evaluate the percentage inhibition of eggs on eggs recovered from *haemonchus. contortus* and *trichostrongylus. columbriformis* in sheep. This test was conducted for evaluation and comparison of the efficacy of albendazole of Chinese, East African Pharmaceuticals, and Indian brands such as those sold on

Ethiopian markets. For efficacy evaluation of these anthelmintic, albendazole of Chinese, East African pharmaceuticals, and Indian brands were purchased from local private veterinary drug stores and prepared in serial concentration in solutions of Dimethyl sulfoxide (DMSO) and distilled water. The eggs of *H. contortus* were adjusted as 100 eggs/0.1ml and 100 eggs were added into 1.5ml test tubes containing various serial concentrations (1.0, 0.5, 0.25, 0.125, and 0.0625µg/ml) of test substances (albendazole) and incubated at 25°C for 48 hr according to guidelines of Belew and Barone [41,44]

Untreated eggs in distilled water were used as a negative control. After 48 hours, the test substances were mixed with one drop of diluted iodine solutions. Then 100µL of the agitated sample was transferred into a Petridish marked with grid and hatched or embryonated, unhatched eggs and dead eggs were counted as described in detailed protocols described by C.elegans model and World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Results were expressed as %inhibition of eggs hatch as a representation of three independent experiments performed in triplicate using the stereomicroscope. The mean number of eggs and larvae at each concentration was calculated and the percentage hatch was derived using the following formula.

$$\text{Percentage hatch} = \frac{\text{Number of hatched eggs}}{\text{Number of unraced eggs}} \times 100$$

The concentrations were expressed in percentage (weight/volume) for each serial dilutions considering that the effectiveness of selected anthelmintic compound is generally higher *in vitro* than *in vivo*, and it was decided to continue the screening of each tested compound at lower concentrations, only if at the highest concentration tested some inhibiting effects were observed in each triplicate of the assays performed in this study according to Ferreira [29].

Larval motility test (LMT): In this method, infective larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* were exposed to an anthelmintic for 24 hours and subjected to test under larval motility test. This test was designed to evaluate the efficacy of ivermectin and levamisole in the small ruminant's nematode according to the procedures described by Demeler [45]. Therefore, ivermectin of Chinese, Indian, and Uruguay and levamisole of East Africa and Indian brands were purchased and tested to compare their efficacy within their brands. For these anthelmintic classes, the tested concentration was the same as used in albendazole. For evaluation of the motility of third-stage (L3) larvae in the presence or absence of increasing dilutions of levamisole and ivermectin of each tested brand, eggs were initially cultured according to the method of Van Wyk and Mayhew [46,47].

Two sheep prepared as donor animals were infected orally with 2500 infective larvae (L3). The infected sheep were checked for the establishment of the infection as of day 21 post-infection using fecal examinations according to Chaka [48]. Then 15 grams of feces with nematode eggs were crushed and homogenized with sterile pestle and mortar. The culture material was moistened daily by spraying distilled water to provide the appropriate

humidity and air to grow the larvae. After incubation for 10 days at room temperature, L3 was recovered by spontaneous migration after downward inverting the culture material and adding warm water. The concentrated larvae were washed three times and the total counted were stored in separate tubes. The storage of counted concentrated larvae was finally diluted and counted three times to distribute the required amount of larvae in each well. Finally, dilutions of counted larvae were adjusted at 50 µL of suspensions containing 50 L3 per 10µL of serial concentrations of ivermectin and levamisole solutions according to guidelines of Dolonska [49]. The plates were then incubated for 24 hours at 27°C and the number of motile and non-motile larvae was counted, particularly focusing on the presence or absence of smooth sinusoidal movement, respectively [50]. The larvae were also incubated in distilled water as negative controls. After taking from the incubator the result was recorded based on the movement of larvae. Therefore, if smooth sinusoidal movement is present, it is additionally stimulated through the exposition of larvae to microscopic light or shaking of well plates. Then results were expressed as % inhibition of larval motility as a representation of three independent experiments performed in triplicate as described by Ferreira and Albonico [29,51].

Adult Motility Test (AMT): Adult motility assay was conducted on mature *H. contortus* worms, collected from abomasa of freshly slaughtered sheep, following the technique of Avinash [43]. It was conducted in petridishes at room temperature (27–30°C). Fifteen worms were exposed in triplicate to each of the following treatments in separate Petri dishes at (1.0, 0.5, 0.25, 0.125, and 0.0625µg/ml) five different concentrations and distilled water alone for a negative control). Accordingly, immediately after the animal’s death, the abomasums were removed, opened, and washed for the collection of adult worms. Then fifteen actively moving adult stages of *H. contortus* were manually picked up from the mucosal surface and the contents of the abomasums, collected and washed in distilled water were placed in petridishes containing tetramisole dissolved in DMSO and diluted in distilled water at final concentrations of (1.0, 0.5, 0.25, 0.125, and 0.0625µg/ml) for efficacy evaluation of tetramisole of Chinese and Indian brands. Again, fifteen (15) adult parasites were placed alone in distilled water for the negative control following the technique of Avinash [43].

The adult parasites placed in petridishes were incubated at 37°C and checked their motility at 2-hour intervals until 10 hours

according to the previous method [29]. Three replications per treatment concentration were employed. After 10 hours, the tetramisole was washed away and the parasites were suspended in distilled water for thirty minutes for the possible recovery of parasite motility. The numbers of motile and immotile worms were counted. The motility and viability of the worms were assessed by gently prodding the worms using a pointed syringe. The response was recorded as either alive or dead. Worms were considered dead when a minimum reaction to touch was observed. Observations were made on the motility or survival of parasites at zero, two, four, six, and eight hours post-exposure (PE) after the beginning of the test under our experimental conditions. Results were expressed as % of motility as a representation of three independent experiments performed in triplicate according to the method of Ferreira [29].

Ethical approval

All experimental procedures involving the use of animals were approved by the Ethics Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Ref No. VM/ERC/18/01/12/2020). Questionnaire surveys were performed after informed consent from participants.

Data management and Analysis

The raw data for the questionnaire survey were collected and inserted into Microsoft excel word office and then analyzed using Statistical Package for Social Sciences (SPSS) version-20 statistical software. Descriptive statistics (percentages) were used to measure the results describing the respondents’ responses to the questionnaire format. Results are presented as percentages and the absolute numbers on which these percentages are in parentheses from a questionnaire survey according to the guidelines of Arsham [24]. The data were analyzed by using R software and SPSS statistical package to test for the significant differences within variables to correlate associated factors in the evaluation of egg hatch inhibition, larval and adult inhibition potential among the tested anthelmintics. A two-way Analysis of variance (ANOVA) was used to detect the significant effect of different anthelmintics on the parasite’s eggs, larvae, and adult parasites in the performance of each in vitro trial. P < 0.05 was considered to be statistically significant. The median effective concentration (EC50) which is the concentration at which 50% of the eggs fail to hatch or the larvae become non-motile as a result of anthelmintic treatment was calculated by using the ‘Quest

Table 2: List of in vitro evaluation techniques of the efficacy of anthelmintic performed against an isolate of *H.contortus* and *T.columbriformis*.

Anthelmintics Tested	Type of brands of tested AH	In vitro test conducted
Albendazole	East African Pharmaceuticals, Ethiopia, Sudan brands	(EHA)
	Chengdu Qiankun veterinary Pharmacy, Chinese brands	
Levamisole	East African Pharmaceuticals, Ethiopia, Sudan brands	(LMT)
	Ashish Life Science Pvt Ltd, Indian brands	
Tetramisole	Chengdu Qiankun veterinary Pharmaceuticals	(AMT)
	Ashish Life Science Pvt Ltd, India	
Ivermectin	Chengdu Qiankun veterinary Pharmaceuticals	(LMT)
	Uruguay, Belgium veterinary drug store	
	Ashish Life Science Pvt Ltd, India	

EHA: Egg Hatch Assay; LMT: Larval Motility Test; AMT: Adult motility Assay

Graph™ EC50 Calculator', an online EC50 calculator tool (<https://www.aatbio.com/tools/ec50-calculator>).

RESULT

Questionnaire survey

The questionnaire survey indicated ivermectin (39 %) was the most commonly used drug followed by albendazole (36 %), tetramisole (17 %), and levamisole (8%). All sheep owners responded that they use anthelmintics only when animals show symptoms like poor body condition, diarrhea, or coughing. Anthelmintics are administered either by prescription from animal health professionals or owners' decisions. Most of the respondents indicated that they selected their drug of choice by color (drug type) and route of administration. The survey indicated that farmers prefer boluses of green color (Albendazole) followed by white (Tetramisole), white but longer in size (levamisole), and subcutaneously injectable (ivermectin) in order of preference. A significantly higher number of respondents administer anthelmintics by animal health personnel (including all ivermectin injections) compared to those who do it on their own (P< 0.05). The frequency of treatment with anthelmintics was, on average, twice per year (Table 3).

In vitro anthelmintic efficacy

Egg hatch test (EHT) for Albendazole brands: In this study, different brands of albendazole utilized by the local farmers of the district were analyzed for their anthelmintic activity against a local isolate of *H.contortus* and *T.columbriformis*

The percentage of eggs that hatch (or conversely die) at each concentration is determined, corrected for natural mortality from control plates, and a dose-response line plotted against drug concentration. For the three tested anthelmintic brands, the egg hatch inhibition potential between treatment and control wells showed a significant difference (**p<0.05**) against both local isolates of *H.contortus* and *T.columbriformis*. Egg hatch test on *T.columbriformis* eggs showed no significant difference between China and India brands of albendazole at 1µg, 0.125µg, and 0.0625µg (P> 0.05). Both brands performed >50% inhibition in egg hatching even at the lowest concentration of 0.065µg. On the other hand, the egg hatch inhibition potential of the East African (EA) brand was much lower than that of the above two brands (P<0.01). It has given egg hatch inhibition of 50% only with 1µg concentration while it remained below this cut-off point for the rest of the drug concentrations (Figure 2). A similar test on *H. contortus* eggs revealed a different performance. No significant difference between China and EA brands of albendazole at 1µg and 0.5µg (P> 0.05) with values of 72 and 78% for China brand and 78% at both concentrations for EA brand. However, from this concentration downward, the EA brand remained below 50% egg hatch inhibition performance whereas the China brand was good (68%) even at 0.065µg (Figure 3).

The linear regression for concentration-response indicates that the EC50 for inhibition of *T.colubriformis* egg hatching by albendazole was 2.375µg, 0.087µg, and 1.199µg respectively for EA, CN, and India brands. The EC50 of Albendazole against *H.contortus* eggs (Figure 4) was 0.504µg, 0.017µg, and 1.886µg respectively for EA, CN, and India brands. Taking EC50 cutoff

Table 3: Responses of farmers to a questionnaire survey on anthelmintic utilization practice

Content of questionnaire format	Responses of the farmers	Percent
Type of commonly used AH	Albendazole	36.0
	Ivermectin	39.0
	Levamisole	8.0
	Tetramisole	17.0
Who administers AH	Professional	79.0
	Farmers by their own	21.0
Frequency per year	One time	12.0
	Two times	51.0
	Three times	32.0
	Five times	2.0
	More than five times	3.0
Source of AH	Private clinic	42.0
	Government clinic	44.0
	Open market	14.0
Alternative if treatment not respond	Change another AH	37.0
	Give other drugs	40.0
	Send to vet clinic	1.0
	Use medicinal plant	10.0
	Sell it to the market	5.0
	Lost after died	7.0
	Total	100.0

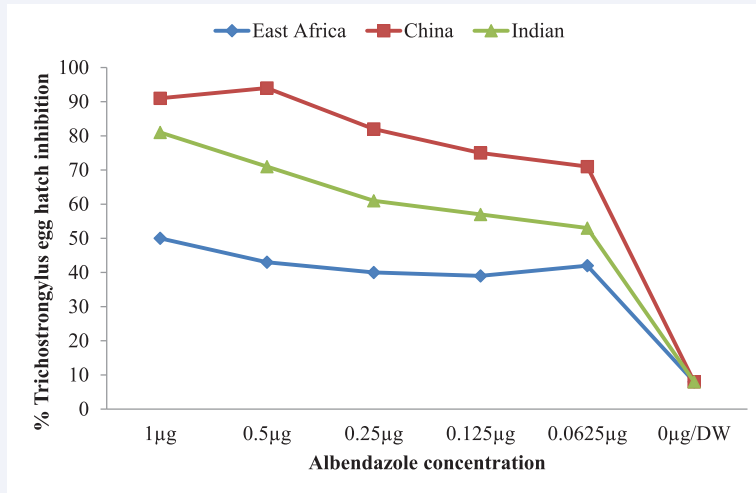


Figure 2 Egg hatch assay performance of different brands of Albendazole against eggs of *T. colubriformis*.

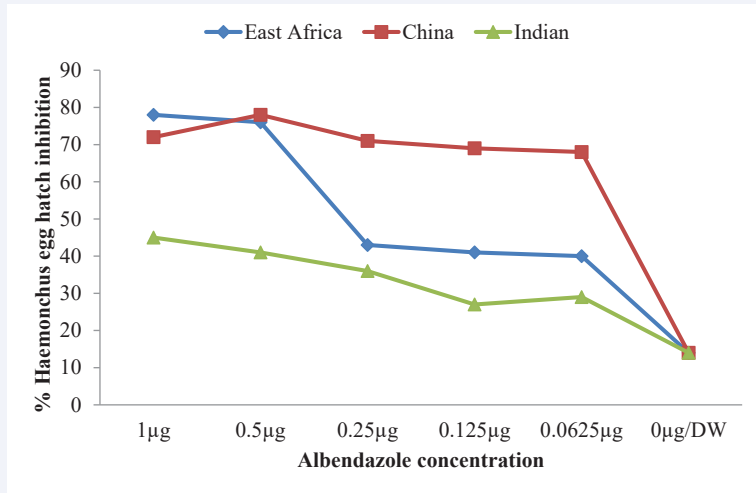


Figure 3 Egg hatch assay on eggs of *H. contortus* with different concentrations of Albendazole brands.

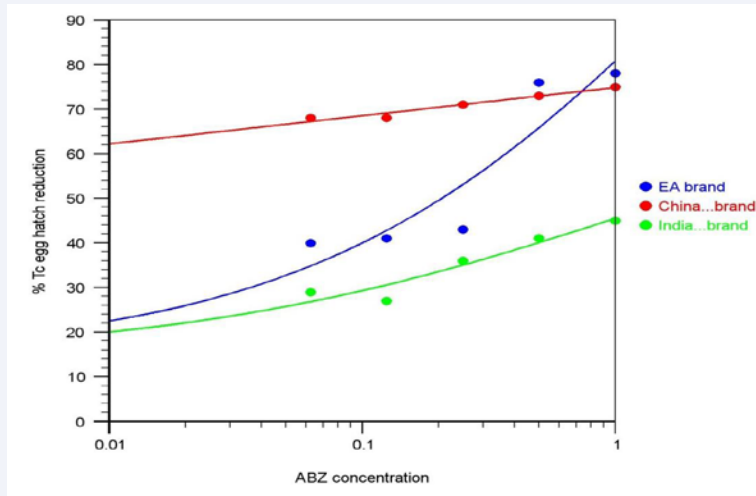


Figure 4 Egg hatch test log-transformed concentration-response curve for Albendazole against eggs of *T. colubriformis*.

value above 0.1µg/ml as an indicator of benzimidazole resistance [33], except the brand from China, the other two do not qualify for dependable efficacy against eggs of both parasites. The transformed drug concentration-response curve for Haemonchus is shown in Figure 5.

Larval motility test (LMT): In this study, larval motility tests with different levamisole and ivermectin brands were conducted. The test can be used to determine the EC50 which is the median effective concentration required to kill 50% of the worm population. It is measured by detecting the presence or absence of sinusoidal movement of larvae in serial concentrations of anthelmintic dissolved in Dimethyl sulfoxide (DMSO). The final concentration of levamisole and ivermectin for each brands were (1, 0.5, 0.25, 0.125, 0.0625 µg/ml). Then data were log-transformed, EC50 values calculated and concentration-response curves created as shown below.

Levamisole efficacy

As depicted on the graph of the Levamisole efficacy test (Figure 6), at 1µg and 0.0625µg concentrations, there is no difference in efficacy between the two brands tested against both *H. contortus* and *T. colubriformis*. East Africa brand of levamisole was significantly more effective at reducing *H. contortus* larval motility than the Indian brand (92% and 76% respectively) at 0.5µg while it is significantly better at 0.5µg, 0.25µg, and 0.125µg against *T. colubriformis* (P<0.05).

The log-transformed concentration-response curve indicates that the EC50 for inhibition of motility of *T.colubriformis* L3 by levamisole was 0.057µg and 4.028µg respectively for EA and India brands (Figure 7), suggesting a much better performance of EA brand over the Indian brand. The EC50 of levamisole against *H. contortus* L3 (Figure 8) was 0.058µg and 0.048µg respectively

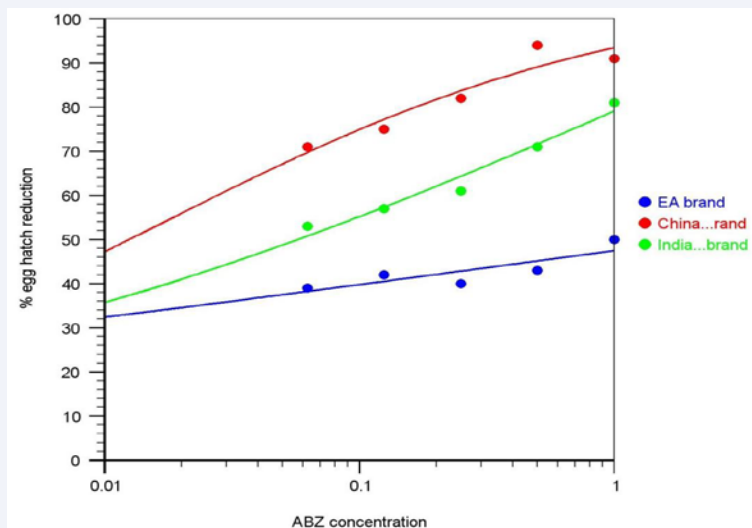


Figure 5 Egg hatch test log-transformed concentration-response curve for Albendazole against eggs of *H. contortus*.

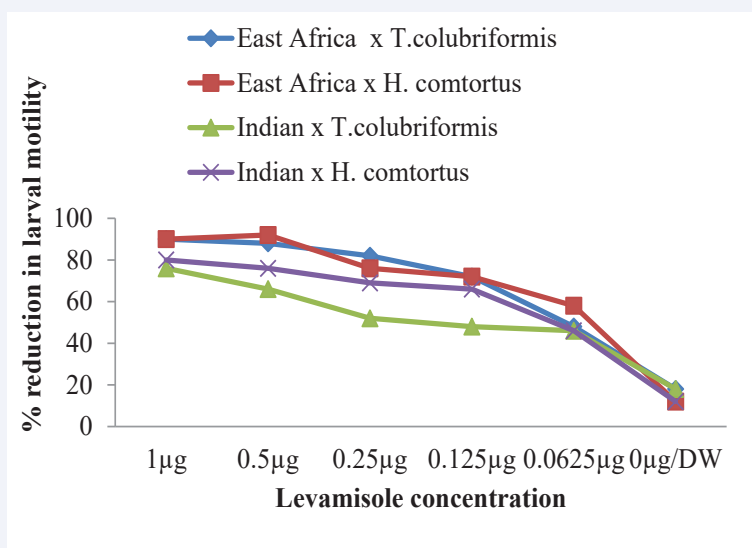


Figure 6 Effect of different brands of levamisole on the motility of *H. contortus* and *T. colubriformis* L3.

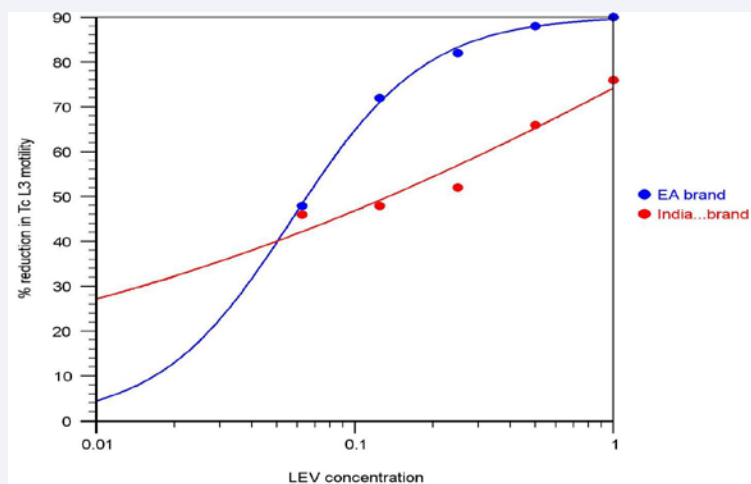


Figure 7 Larval motility test log-transformed concentration-response curve for levamisole against eggs of *T. colubriformis*.

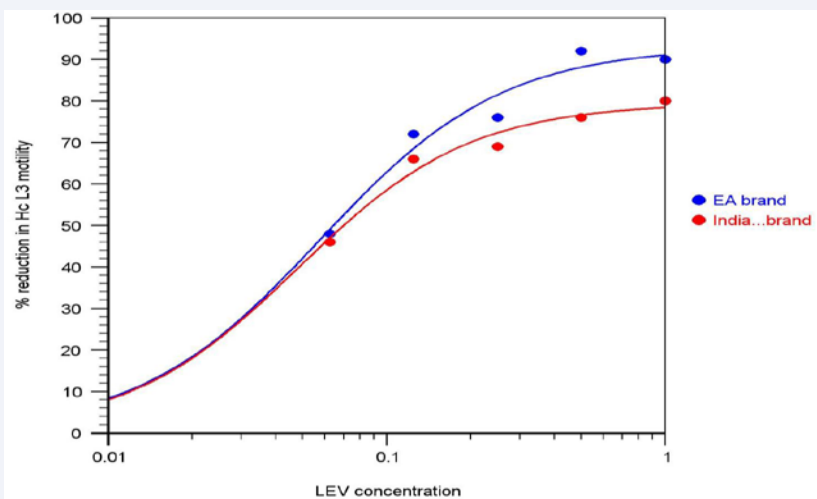


Figure 8 Larval motility test log-transformed concentration-response curve for levamisole against eggs of *H. contortus*.

for EA and India brands; both being in the range of good efficacy at 0.1 μg cutoff point.

Ivermectin efficacy

Except for a higher reduction in L3 motility by China brand of ivermectin at 1 μg, there was no significant difference between the three brands (China, Uruguay, and India) of the drug against *T. colubriformis* L3 with all other concentrations. On the other hand, the three brands of ivermectin at all concentrations tested had similar efficacy against the motility of *H. contortus* L3 (Figure 9). At 0.5 μg China and India brands were better than Uruguay brands ($P < 0.05$) on the same parasite. China brand also had better performance than Uruguay brand at 0.25 μg and 0.125 μg concentrations ($P < 0.05$).

The linear regression for the concentration-response curve indicates that the EC₅₀ for inhibition of motility of *T. colubriformis* L3 by ivermectin was 0.073 μg, 0.178 μg, and 0.040 μg respectively for China, Uruguay, and India brands (Figure 10), suggesting a lower performance of the Uruguay brand compared to the others.

Similarly, the EC₅₀ of ivermectin against *H. contortus* L3 (Figure 11) was 0.025 μg, 5.040 μg, and 3.061 μg respectively for China, Uruguay India brands; only the first one being in the range of good efficacy at 0.1 μg cutoff point.

Adult motility test (AMT): China and India brands of tetramisole were tested against Adult *H. contortus* worms using adult motility test by incubating at 37°C for ten hours of incubation time. Then the effect of tetramisole on adult parasites was analyzed to compare their anthelmintic activity in inhibition/killing of the parasites (Figure 12). In this regard, both brands of the drug have performed similarly excepting at 0.25 μg/ml where the brand from China was significantly better at killing the adult parasite ($P < 0.05$). The log-transformed treatment response indicates that the EC₅₀ for inhibition of motility of adult *H. contortus* by tetramisole was 0.134 μg and 5.576 μg respectively for China and India brands (Figure 13), suggesting a marginal performance of the former and much lower efficacy of the latter brand.

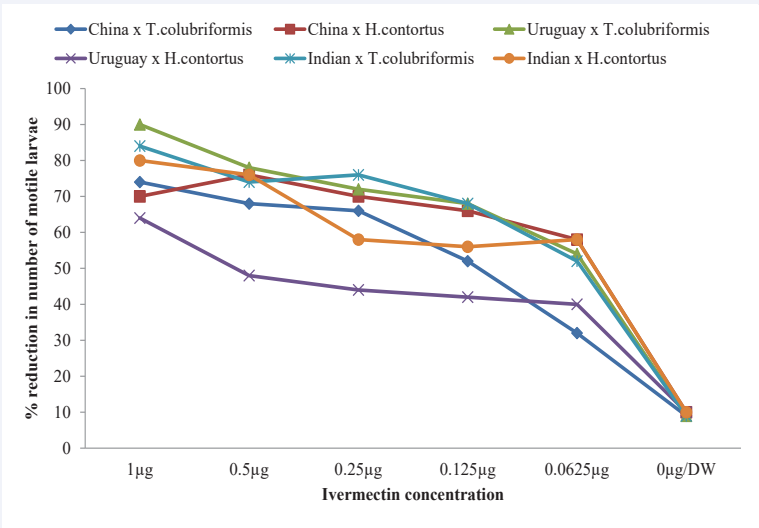


Figure 9 Effect of different brands of ivermectin on the motility of *H. contortus* and *T. colubriformis* L3.

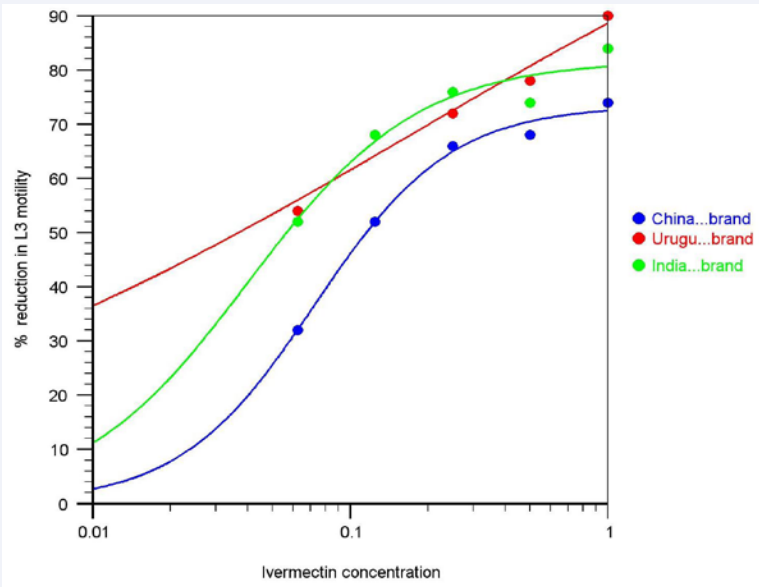


Figure 10 Larval motility test log-transformed concentration-response curve for three brands of ivermectin against eggs of *T. colubriformis*.

DISCUSSION

Anthelmintic utilization practices

Anthelmintic treatments such as Albendazole, Ivermectin, Levamisole, and Tetramisole are used by the farmers residing in and around Bishoftu to treat parasitic infections in ruminants. According to Adediran [52], it has been observed that frequent use of the same group of anthelmintic, use of anthelmintics in suboptimal doses, prophylactic mass treatment of domestic animals, and frequent and continuous use of a single drug in a given area contribute to the widespread development of anthelmintic resistance [27]. In this study, the result of the questionnaire survey indicated that Ivermectin was the most widely used anthelmintic followed by Albendazole, Tetramisole,

and Levamisole respectively. This is contrary to many other reports which have demonstrated that easily administrable anthelmintics in the form of Bolli are more preferred than injectable ivermectin [1,25,53]. Such variation could be attributed to access to formal veterinary services. In and around Bishoftu, there are several on-station and mobile veterinary service providers. Ivermectin is commonly given because it attacks both internal and external parasites [54]. A similar study eight years back in the same locality, reported Albendazole followed by ivermectin were drugs of choice [25], suggesting that there have been shifts over time. This is supported by the fact that most questionnaire survey respondents affirming anthelmintic drug administration by animal health personnel.

Although the majority of them get drugs from formal veterinary

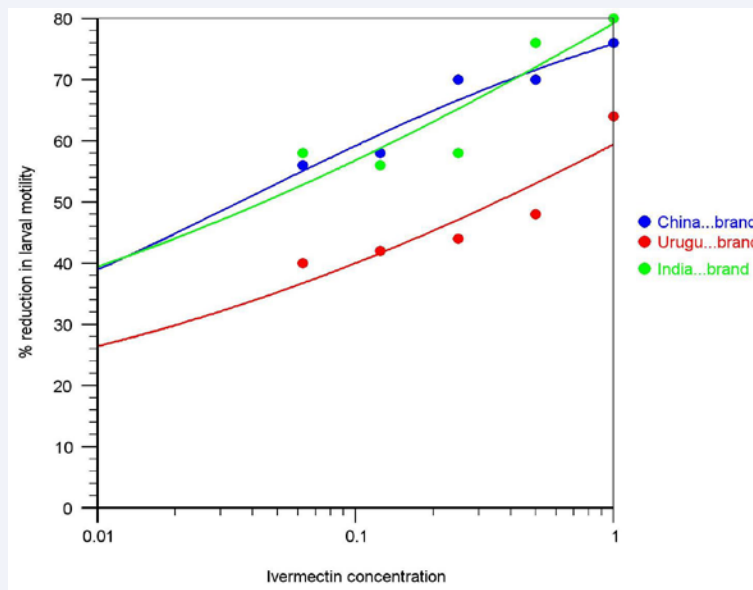


Figure 11 Larval motility test log-transformed concentration-response curve for three brands of ivermectin against eggs of *H. contortus*.

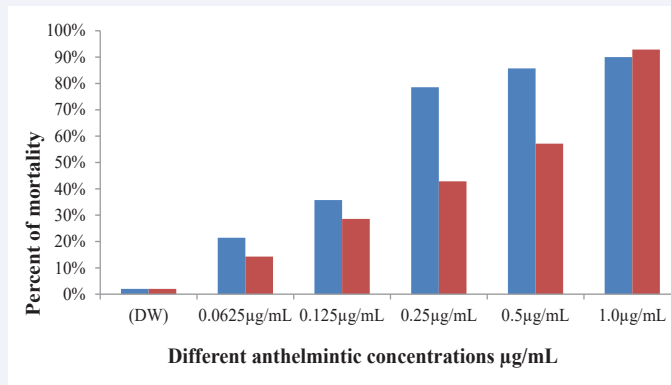


Figure 12 Effect of two brands of tetramisole on adult *H. contortus* motility/survival during the adult motility test.

service providers or drug stores, 14% of the respondents indicated purchase of anthelmintics from open markets suggests that mishandling and misuse of the commonly used anthelmintics are possible. This observation also agrees with those from previous works by Kumsa and Nurfeta [25,47,53,55]. Forty-four percent of respondents stated that they have dewormed their sheep twice a year; while 51% of owners treated them three times a year. This is in line with previous findings [47,53,56], elsewhere in Ethiopia. Although this frequency is acceptable on an individual animal basis, the fact that few anthelmintic groups are consistently utilized in the area indicates the parasites circulating in the area are continuously exposed to these drugs and hence the risk of anthelmintic resistance is evident. Most of the respondents indicated that their animals displayed improvement on both clinical signs and body condition after treatment. This supports the report of Datiko [47], who stated that 81% of the respondents indicated that their animals have shown improvement in both clinical signs and body condition after treatment.

Efficacy of anthelmintic brands circulating in Bishoftu

It is very important to monitor anthelmintic efficacy at regular intervals to detect subtle changes, as early as possible to avoid the establishment of anthelmintic resistance [57]. This may be performed by accessible and sensitive *in vitro* diagnostic tests, *in vivo* tests on animals, and other techniques. This ultimately enables the choice of an anthelmintic for therapeutic use in the field [49,58]. In this study, *in vitro* egg hatch inhibition, larval motility, and adult motility assays were done with different brands of albendazole, ivermectin, levamisole, and tetramisole against a local isolate of *H. contortus* and *T. columbriformis* and interpreted according to WAAVP recommendation [38,41].

Accordingly, all tested anthelmintics brands of the drugs showed a dose-dependent inhibition of egg hatching on both *T. colubriformis* and *H. contortus*. This result is consistent with the findings of Várady [57] and Belew [41]. However, based on their EC50 (the median drug concentration that produces the desired effect in 50% of the test eggs/larvae/adult and the cutoff value

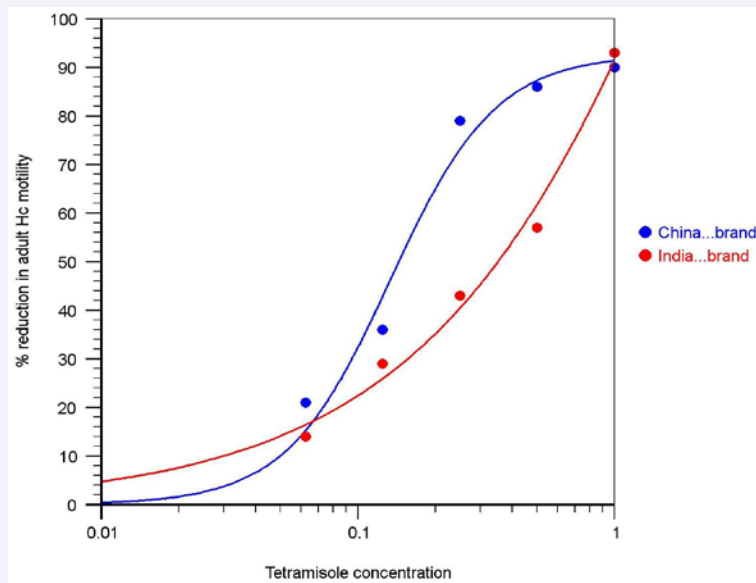


Figure 13 Larval motility test log-transformed concentration-response curve for three brands of ivermectin against eggs of *H. contortus*.

of 0.1 µg for efficacy, the brands vary in their effectiveness. China brands (already described in Table 8) were effective against eggs of *T. colubriformis* and *H. contortus* (Albendazole), L3 of the two parasites (Ivermectin), and marginally effective against adult *H. contortus* (tetramisole). These values are also well below the commonly accepted cut-off value for BZ-resistance of 0.1 µg TBZ/ml [33]. A similar result to benzimidazole agents (Greece) has already been demonstrated on susceptible isolates of *H. contortus* obtained from the Ogaden region (Ethiopia) [17]. On the other hand, Albendazole from East Africa and Indian brands require much higher doses to produce the desired efficacy suggesting that they are of inferior quality. Ivermectin from the China brand and levamisole from the East Africa brand are both effective against L3 of the two parasites *in vitro* whereas Indian brands of Levamisole and ivermectin were respectively effective on *haemonchus. contortus* L3 and *trichostrongylus. colubriformis* indicating the need for such information while selecting the best anthelmintic of choice. The relative finding was reported against *haemonchus. contortus* for the half-dose IVM, half-dose ABZ, full-dose ABZ showed that *haemonchus. contortus* is resistant to half-dose IVM and ABZ. Full-dose IVM was effective against *H. contortus*. The combined treatment was more effective against *haemonchus. contortus* than ABZ alone.

Generally, better efficacy of anthelmintic brands could be attributed to the high quality of the drugs or the low frequency of anthelmintic treatment practice in the area as similarly reported with some *in vivo* studies made on the susceptibility of *H. contortus* by Keyyu [59] and Chaka [48]. However, Kumsa and Abebe [12], reported resistance of *H. contortus* to different anthelmintics. The variation in efficacy of anthelmintic agents may indicate the spectrum of treatment practice or use of anthelmintic agents, introduction of resistant worms with livestock from other farms, or selection of resistant strains in some localities.

CONCLUSIONS AND RECOMMENDATIONS

This study was initiated to assess farmers' anthelmintic utilization practices on sheep and goats in the Ada'a district and evaluate the efficacy of locally available anthelmintic brands using three *in vitro* tests. Ivermectin followed albendazole; tetramisole and levamisole were the most commonly used anthelmintics in the study area. Some practices potentially predispose the available anthelmintics to drug resistance. Dose-dependent inhibition of egg hatching, larval and adult motility were observed in all *in vitro* tests performed. The Egg hatch assay showed higher performance of the albendazole brand from China compared to those from East Africa and Indian manufacturers. Similarly, the larval motility test suggests that based on their EC50, both levamisole and ivermectin from china have good efficacy against L3 of *T. colubriformis* and *H. contortus* whereas only levamisole of East African origin was effective against L3 of both parasites. Levamisole of Uruguay, Levamisole, Ivermectin, and Tetramisole of Indian origin was not effective or only partially effective against the two species of parasites as per the different assay type. The lower efficacy of some of the brands could be attributed to their quality or other factors that are worth investigating in the future.

Based on the above conclusion, the following recommendations are forwarded:

- Farmers should continuously be educated on the risks of anthelmintic resistance and the need to utilize formal veterinary services in their vicinity
- Veterinary service delivery should be further improved to address all farmers at their convenient location
- Further research is needed on testing the quality of anthelmintic drugs being imported and circulating in the area and beyond
- Those brands that have been proven effective *in vitro* should be further tested *in vivo*

- The few brands that were tested and proven to be good should be used strategically and carefully to minimize the risk of the development of resistance.

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