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In vitro egg hatchability inhibition effect of *Moringa oleifera* leaf aqueous and ethanol extraction against *Strongyles* type egg of ovine in arsi zone, Southeastern Ethiopia

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Abstract

Livestock is an important prospective sector that contributes to solving problems of all farmers of Ethiopia and Africa by poverty alleviation. Meanwhile, this species faces several problems including widespread resistance and significant economic losses to commercial anthelmintic, besides drug residue and toxicity problems so, alternative strategies of parasite control, with natural anthelminthic plants properties, were mandatory.For this reason, an experimental study was conducted from November 2019 to April 2020, in Asella regional veterinary laboratory with aim of estimate the inhibition of egg hatchability effect of aqueous and ethanol extraction of *Moringa oleifera* leaf against ovine *Strongyles-type eggs* compared to Albendazole. In this study, the purposive sampling method was implemented based on animals that suspected with parasites and the sample was collected directly from the rectum. Four different concentrations i.e,1.5, 2, 5, and 8 mg/ml both aqueous and ethanolic extracts for 48 hours for the case of fresh egg and 6 hours for embryonated egg were prepared and tested through the egg hatch essay test. This result shows in terms of the mean inhibition percentage of embryonated egg and fresh egg hatch 99.8%±0.7% and 100%±1.41% egg hatching at 8 mg/ml with anLC50 value of 2.042 mg/mland 2.04mg/ml respectively. The aqueous extract also induced 95.23%±0.47% and 98.81%±7.77% inhibition of embryonated and fresh egg with LC50 values of 2.63 and 2.45 mg/ml respectively. The *Moringa oleifera* leaf aqueous and ethanolic extracts have a potential anthelmintic activity comparable to Albendazole. Therefore the ethnoveterinary of Ethiopia should focus on this multi-functional plant with further in vivo and in Vitro study will necessary to validate the anthelminthic property of this plant.

Keywords: *Anthelminthic, Moringa oleifera, Ovicidal, S. ovine egg, Tiyo district, Ethiopia*

1. Introduction

Livestock is an important prospective sector that contributes to solving problems, sources of income, and poverty alleviation worldwide (Millar and Photakoun, 2008). In the varied agro-climatic zones of Ethiopia, small ruminants are an important source of income for rural communities and are one of the nation's major sources of foreign currency from exports (Tolossa, 2014and Allaie *et al.*,2018). Meanwhile, that species faces several problems including parasitism like strongylosis, but their impact is greater in sub-Saharan Africa in general and Ethiopia in particular due to the availability of a wide range of agro-ecological factors suitable for diversified hosts and parasite species (Derso and Shime, 2017). 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 (Lamy *et al.*, 2012).

A huge amount of money is spent annually worldwide to combat helminth parasites in livestock, the principal mode of control is based on the repeated use of commercial anthelminthic drugs such asbenzimidazole, imidazothiazole, and ivermectin groups are often used for this purpose even if, nowadays their effectiveness is compromised due to parasites resistance (Allaie *et al*., 2018). Moreover, toxicity due to inappropriate dose administration and the risk of drug residues in animal products are other big problems associated with the use of synthetic drugs (Mund *et al.*, 2017).

To solve this kind of problem medicinal plants are considered as an alternative source of compounds that are biodegradable into non-toxic products and sustainable methods readily adaptable to rural farming communities (Tayo*et al*., 2014).From this *Moringa* is an important medicinal natural herb referred to as a miracle tree as almost every part can be used for food or has some other beneficial properties (Daba, 2016). In the tropics, it is used as forage for livestock, and in many countries; it is used to treat various ailments (Ashfaq*et al.*,2012).There are so many inventions and research works done on *Moringa*due to its high activity and fewer side effects (Normile,2003).Conversely, Very little works have been performed in our country to investigate the anthelmintic properties of Moringa plants on livestock. For this reason, the present study has taken great impetus. This research work provides useful information on egg hatchability inhibition effect of *Moringa oleifera* leaf aqueous and ethanol extraction against *strongyles* egg of ovine.

1.1 General Objective

The general objective of this study was to investigate the egg hatchability inhibition effect of *Moringaoleifera* leaf aqueous and ethanol extraction against *strongyles egg* of ovine.

1.1.1 Specific objectives

- To determine in vitro ovicidal activity of *Moringa* leaf aqueous extracts against *strongyles egg* of ovine
- To determine in vitro ovicidal activity of *Moringa* leaf ethanol extraction against *strongyles egg* of ovine
- To determine the evaluability of *Moringa* leaf drug actions on *strongyles egg* and to come up with the scientific drug resistance and cost-effectiveness.
- Provides means to rapidly screen for plant extracts and to analyze the possible mechanisms involved in the interactions between active compounds and parasites.

2. Materials and Methods

2.1Study Area

The experimental study was conducted in Asella regional veterinary laboratory from November 2019 to April 2020. Asella town is located in Oromia region. The town, which is the capital of Arsi zone, is located at about 175 km Southeast of Addis Ababa at 6°59' to 8°49' N latitudes and 38°41' to 40°44' E longitudes with an altitude of the area ranges from 2500 to 3000 meter above sea level. The agricultural production system of

the study area is of mixed crop and livestock production. The area is characterized by mid subtropical temperature ranging from 5°C-28°C and with relative humidity ranging from 43 to 60%. The annual average rainfall is 1200 mm and mostly with clay type of soil and in rare case black soil. The area has a bimodal rainfall occurring from March to April (short rainy season) and July to October (long rainy season). The area covers 23674.72 km square and topographically has highland escapement and lowland areas. The area is densely populated, with a livestock population of 85,893 cattle, 57,118 sheep, 10,725 goats, 7841 horses, 15,642 donkeys, 517 mules, and 35,489 poultry. The farmers in the ;area practice a mixed crop-livestock farming system. The high land areas are found centrally and the low lands dominate the periphery of the area (APEDO, 2007)

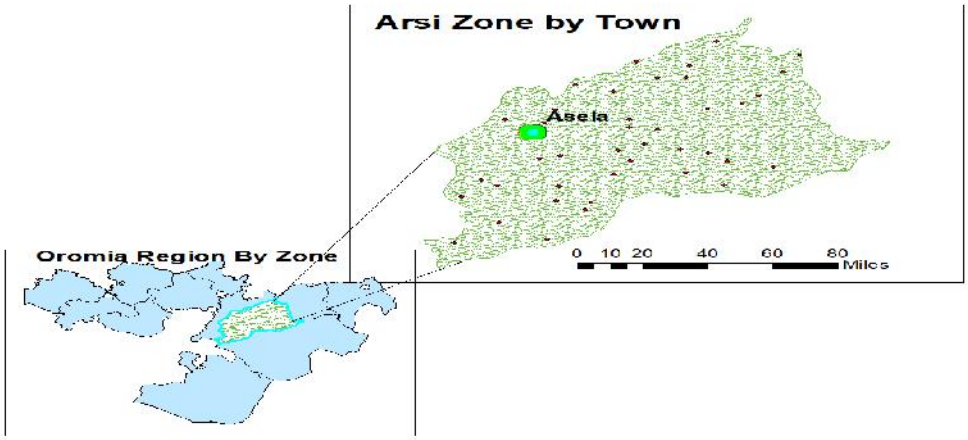

Figure 1: Map of Study Area

2.2 Study Design

The experimental study design was conducted, due to in the fact that to comparison the *Moringa olifera* leaf extract with that of commercial drug Albendazole with the help of different concentration of Moringa leaf extract to come up of the cost-effectiveness and drug resistance of the parasite that occurs in grazing animals. It may show future researchers to conduct more activities concerning these types of natural drug-using from herbal drug purifying and distributing to the worlds.

2.3 Sampling Techniques and Sample Size

The sampling techniques would be conducted with purposive sampling method from naturally infested ewes that coming to Tiyo district veterinary clinic from the rectum after confirming that the animals had not been treated with any anthelmintic agent for at least four weeks before the study. The required sample size for the study was determined by the formula given by (Dell *et al.*, 2002) based on the expected prevalence (50%) of *Strongyles* and the 5% desired absolute precision and 95% confidence interval. The following formula was used to determine the sample size.

$$n=\log\beta/\log\rho$$

Where:
n = sample size
ρ = proportion of the animals that are not infected
β=5% desired absolute precision

$$n=\log 0.05/\log 0.5=4.333$$

Thus five animals should be examined to have a 95% chance of detecting an infection that has affected 50% of the animals in the population.

2.4 Collection and Storage of Plant Material

Fresh leaves of *Moringa oleifera* were collected around Asella city and transported to Asella regional veterinary laboratory. The collected leaves were, washed thoroughly and dried under shade,at ambient temperature for three weeks. Dried leaves were ground to powder and stored in airtight plastic bags in the laboratory until using (Wabo Pone*et al.,* 2010).

2.5 Plant Extracts Preparation

Two types of extracts (aqueous and ethanolic) were prepared to compare their activities. For ethanolic extract, the procedure used was as briefly described by (Wabo Pone*et al.,* 2010).Fine powders were macerated in the ration of dry powder to solvent (1:5) for 72hr to soften and break the plant's cell wall to release the soluble phytochemicals. The mixture was daily stirred and 72 hours later, this solution was filtered throug h a 100 mesh (O. 15 mm aperture) in side beaker and after filtrate by mesh it also filtrate with Whatman filter paper (No. 1: 125mm). This was done by hanging of highland for 24hours.After all this the filtrate was evaporated to dryness in an air oven at 40°C.After complete solvent evaporation, the solid filtrate was stored in capped labeled bottles and kept in the refrigerator at 4°C until use (Bagavan *et al.,* 2009).For the aqueous extract, a procedure similar to that of ethanol extraction was applied except that the distilled water was used and the maceration took 48 hours (to avoid the growth of fungi). Also, the drying of the solvent was done in 24 hours in a ventilated oven heated at 50^{o}c.

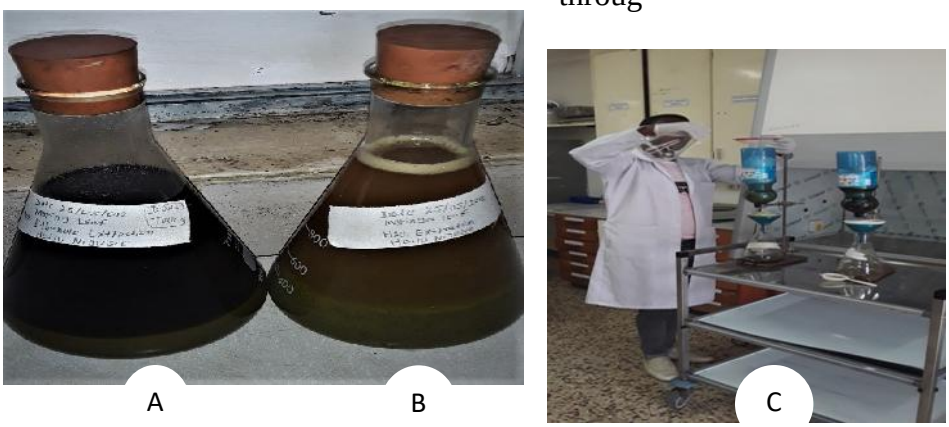

Figure 2:**A.** Ethanol extraction **B**. Water extraction and **C**. Filtering the mixture using filter paper.

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The MIC will carry out by preparing the dried plant extract in different concentrations. 80mg dried extract and Albendazole brought from a local pharmacy was diluted with 10 ml distilled water to obtain a stock solution of 8 mg/ ml to prepares four different solutions of concentrations, 1.5, 2, 5, and 8 mg/ml to evaluate the egg hatch inhibition efficacy of *Moringa leaf* extracts.

2.7 In-vitro Anthelmintic Activity

In vitro, the anthelmintic activity of the plant extract is evaluated again *strongyl type eggs* were using an egg hatch test (EHT).

2.7.1 Egg hatch test (EHT)

The test procedure followed that recommended by the World Association for the Advancement of Veterinary Parasitology WAAVP (Coles *et al.*, 2006). The eggs were extracting by sieving, centrifugation, and flotation in saturated sodium chloride. Then *strongyle-type eggs* were counted using the McMaster. Aqueous and alcoholic extracts of the plant materials were used as active treatment. Albendazole (pure standard reference) was used as positive control while untreated eggs in water were used as a negative control. Approximately 100 freshly collected eggs (1 ml egg suspension) were added per 5ml test tubes and mix with the same volume at different concentrations of plant extract and Albendazole. Aqueous and ethanol extracts of the plant materials were used as active treatment. Albendazole (pure standard reference) was used as positive control while untreated eggs in distilled water were used as a negative control (Ahmed *et al.*, 2020).The test tubes were incubated at room temperature (27°C) for about 48 hours. Unhatched eggs, as well as embryonic eggs in each of the test tubes, were counted.

The same number of un embryonated eggs were addedper test tube as above,as allowed to stand at room temperature for about 24hr until the eggs developed into a fully embryonated pre-hatched stage.when the first stage larvae became transparent and started moving actively within control test tubes 1ml of prepared range of product concentration was added to each test tube .the test tubes then covered and incubated for a further 6hr at room temperature to allow for almost complete hatching. Then all the embryonated eggs and first-stage larvae were counted.

2.8 Statistical Analysis

Comparison of the mean inhibition percentage of egg embryo nation and mean inhibition percentage of egg hatch at different concentrations with control was performed by one-way analysis of variance (ANOVA) using Tukey HSD multiple comparison test. Statistical analysis was performed by using the software SPSS version 20.0. Results were deemed statistically significant if $p< 0.05$ at 95% confidence intervals. The 50% inhibitory concentration (IC50) of the hatchability of the eggs and embryonated inhibition was determined using the regression lines of the probit according to the decimal logarithm of the concentrations.

3. Results

The data revealed that ethanolic extract of *Morinaga oleifera* leaf showed better activity against egg hatchability of *strongly type egg* of nematode than aqueous extraction. The results were comparable with that of the reference drug of 300mg commercial Albendazole ($p > 0.05$). The efficacy of *M. oleifera* leaf extracts in inhibiting egg embryonation of *strongyle type egg* at different concentrations is presented in (**Table 1**). From this table, we observed that negative controls (distilled water) did not affect egg embryonation while *M. oleifera* extracts inhibited embryonation in a concentration-dependent fashion.

Ethanolic extract was efficient 32.64%±11.07%at 1.5mg of egg embryonnation, reaching 99.8%±0.7%at 8mg/mlwith significant difference ($P < 0.05$).This result was statistically comparable to the 100% efficacy of 300mgalbendazole ($p > 0.05$). Aqueous extracts also inhibited embryonation with mean efficacy of 17.02%±20.97%and 95.23%±0.47%at 1.5 and 8mg/ml respectively with significant difference ($P < 0.05$). The IC50s calculated from equations of regression lines of probit according to the decimal logarithm of concentrations (**Fig 4**) were 2.63 and 2.042mg/ml for aqueous and ethanolic extract respectively.

(**Table 2**) indicates the activity of the extracts in inhibiting the fresh *strongyl egg* from hatching at increasing doses. Just like an embryonated egg, distilled water did not influence the egg hatch while the extracts exhibited concentration-dependent activity**.** Aqueous extract and ethanol extract inhibited more than 96% egg hatch at 5 mg/ml, reaching 98.81%±7.77%and 100%±1, at

8mg/mlrespectively.IC50s values 2.45 and 2.04mg/ml were obtained for aqueous and ethanolic extracts respectively from equations of regression lines of the probit of egg hatch inhibition according to the decimal logarithm of concentrations (**Fig 5**).

In the ovicidal assay, the ethanolic extract exhibited the highest or 100%±1.41% and99.8%±0.7%efficacy of egg hatch inhibition and egg ebmbryonation inhibition at 8mg/ml respectively. This result was statistically comparable to the 100% efficacy of 300mg albendazole ($p > 0.05$). Interestingly, at least 90% of the inhibited eggs failed to develop into larva, and approximately 10% contained larva that failed to hatch (data not shown in the table). For example,(40× magnification) out of 99.8% eggs that were inhibited at 8 mg/mL ethanol extraction, 93.50% remained morulated as shows (**Fig 3 B**) while 6.5% shows 'larva failing eclosion' as showed (**Fig 3 A)**. On the other hand, the aqueous extract of *M. oleifera* leaf effectively inhibited egg hatching by 98.81%±7.77%at 8 mg/ml, in which 85.67% of the inhibited eggs remained morulated while 14.33% showed 'larva failing eclosion'. At least 80% of the unhatched eggs in all aqueous extracts failed to form larva or remained morulated. The positive control or albendazole showed 100% ovicidal activity by inhibiting the formation of the larva inside the egg at 5mg/ml.

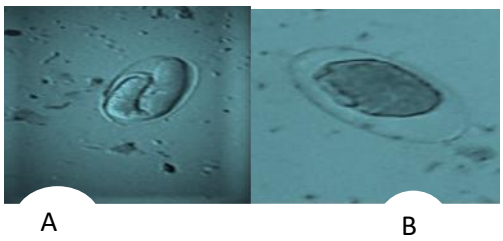

Figure 3: Egg hatch assay of *strongyle type eggs* exposed to *M.oleifera* leaf extracts after 48hrshowing **A.**Larva failing eclosion and **B**. Morulated eggs.

Table 1: Mean inhibition percentage of egg embryonation ± standard deviation of *Moringa oleifera* leaf extracts at different concentrations against *strongyles* egg of ovine.

Concentrations (mg/ml)	Ethanol extraction Mean±sd	Water extraction Mean±sd	Albendazole Mean±sd	D.water Mean±d
1.5mg	32.64%±11.07%	17.02%±20.97%	91.42%±7.83%	NA
2mg	44.68%±8.25%	29.47%±18.8%	92.87%±6.9%	NA
5mg	95.2%±1.78%	93.61%±0.24%	100%±0.94%	NA
8mg	99.8%±0.7%	95.23%±0.47%	100%±0.24%	NA

Legend: Sd= standard deviation, NA=Not applicable, D.water=distilled water

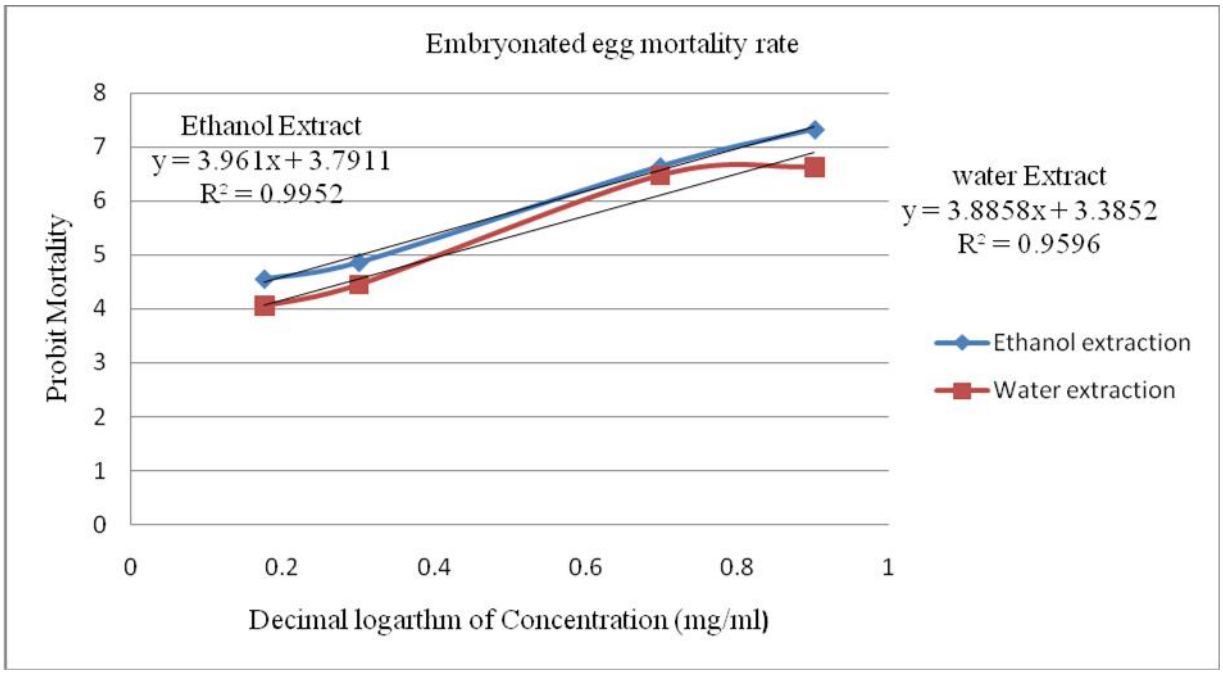

Figure 4: Evolution of probit of embryonated egg mortality rate according to the decimal logarithm of concentrations of *Moringa oleifera* extracts.

Table 2: Mean inhibition percentage of egg hatch ± standard deviation of *Moringa oleifera* leaf extracts at different concentrations against *strongyles egg* of ovine.

Concentrations (mg/ml)	Ethanol extraction	Water extraction	Albendazole	D.water
	Mean±sd	Mean±sd	Mean±sd	Mean±sd
1.5mg	26.43%±1.88%	17.24%±1.03%	96.6%±2.5%	NA
2mg	40.23%±1.65%	22.98%±2.83%	97.7%±4.2%	NA
5mg	99.1%±4.24%	96.55%±3.38%	98.9%±9.2%	NA
8mg	100%±1,41%	98.81%±7.77%	100%±0.0%	NA

Legend: Sd= standard deviation, NA=Not applicable, D.water=distilled water.

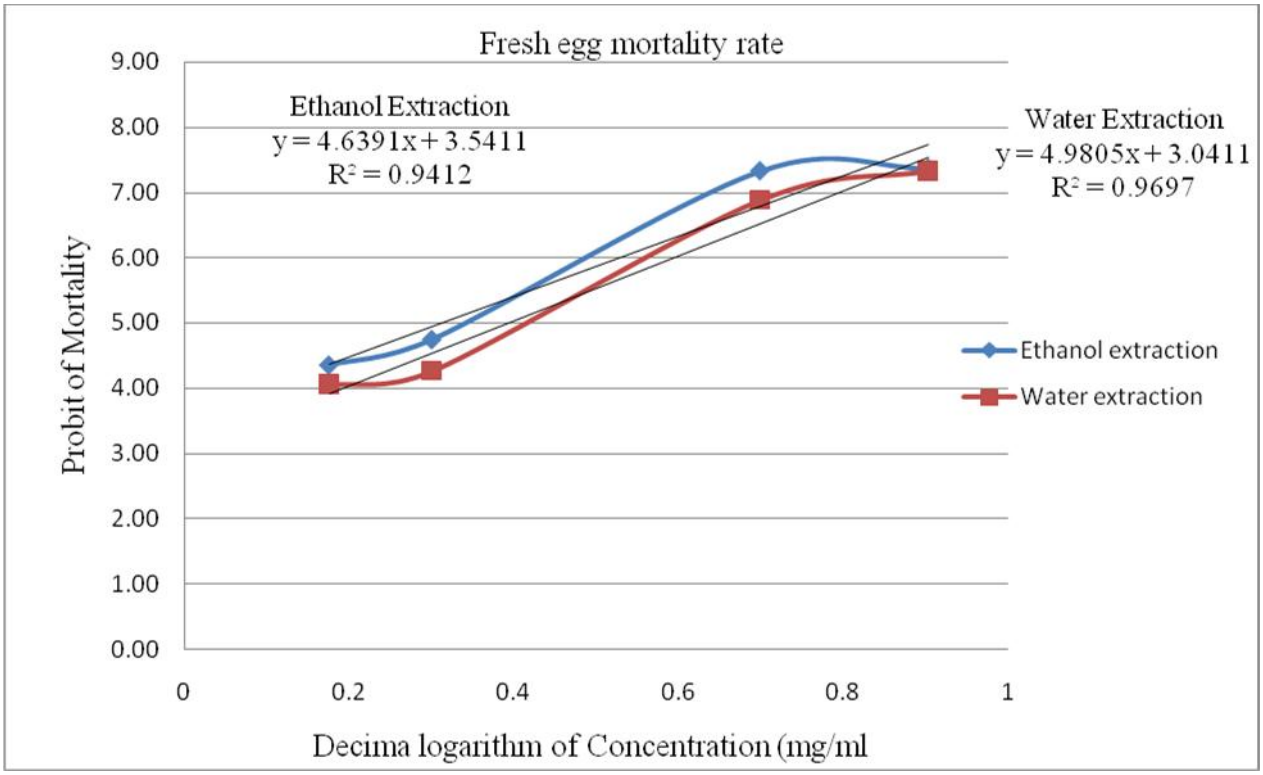

Figure 5: Evolution of probit of the fresh egg mortality rate according to the decimal logarithm of concentrations of *Moringa oleifera* extracts

4. Discussion

In developing countries, the identification of a plant with an anthelminthic property may help to build an integrated and sustainable approach for the control of gastrointestinal nematodes in small ruminants (Githiori *et al.*, 2014).*M. oleifera* was a miracle tree which reported to contain various bioactive compounds with pharmacological activities such as anthelmintic property. The objective of this study was to evaluate in vitro ovicidal efficacy of aqueous and ethanolic leaf extracts of *M.oleifera* against *strongyle type eggs*. In this study, *M. oleifera* leaf extracts presented a concentration-dependent activity against egg hatchability and embryonation, suggesting that an increase in the concentration of plant extract is followed by a supplementary input of different active compounds.

Aqueousand ethanolic extract of *M. oleifera* presented comparable activity on egg's hatchability efficacy. The first extract (infused) inhibited 95.23%±0.47%and 98.81%±7.77%of egg embryonation and egg hatch at 8mg/ml respectively. While the latter (ethanolic) inhibited99.8%±0.7% and100%±0.24%of egg embryonation and egg hatch at 8mg/ml respectively. Tayo *et al.*(2014)recorded the same observation between crude aqueous and ethanolic extracts of *Moringa oleifera* on *H. contortus*. They proved that inhibition of *H. contortus* egg embryonation could be induced by using of aqueous extract of *M.oleifera* leaf extracts by (94.5% ± 4%) and (92.8% ± 6.2) at 5mg/ml for the ethanolic extract.Also, they recorded inhibition of *H. contortus* egg hatchability by(90.2% ± 8.4%) for the aqueous extract and by (99% ± 2%) for the ethanolic extract at a concentration of 5 mg/ml. This inhibitory activity was concentration-dependent as described in this paper.

In a related study, the ovicidal activity of *M. oleifera* seeds is moderately effective (80–89% efficacy) using the aqueous extract and effective (90–98% efficacy) using the ethanolic extract at a concentration of 15.6 mg/ml extract (Cabardo and Portugaliza, 2017).

On their phytochemical screening, they detected tannins in the ethanolic extract and saponins in Aquas extraction of *M. oleifera* seeds. They concluded that these bioactive substances are implicated in some plants for ovicidal and larvicidal activities against helminths. In the same way, Ali *et al. (* 2011) reported that Saponins as an excellent source of cytotoxic and penetrated the different layers of the egg and inhibited the formation of the larva by affecting the morula. Besides Cabardo and Portugaliza(2017) had demonstrated that saponins and tannins could stop the larval formation and hatching process of *H. contortus* eggs. Also, other research said that tannins could bind with feed nutrients and possibly prevent bacterial growth in the feces (larva feed on bacteria) and so limit the feed available for larval growth, or in some other way inhibit larvae formation in said egg and have ovicidal activity (Belay *et al*., 2013).

Aside from the ability to stop the larval formation and ovicidal activity this research also detected a small percentage of the affected eggs forme larvae inside eggs that failed toeclosion andeggs that remained as morulated.Mechanisms of plant extract inhibited hatching of the egg with the full-grown larva and failer of larvae eclosion were outlined byVargas-Magaña *et al.(*2014). They reasoned three mechanisms: first, the extract affects the permeability of the eggshell; second, the extract inhibits some enzymes for egg hatching; and third, the extract affects the hatching receptors found in eggshells.

The results of this research suggested that the two extracts seem to be more active on hatching than embryonation mechanism. The blastomere of Unembryonated eggs found in solutions containing the two extracts was destroyed. The probable reason for the minor differences between aqueous and ethanolic extracts could be due to variation in the solubility of a bioactive compound in a different solvent. The ovicidal activities observed in this study with different extracts may be attributed to the presence of saponins, steroids, carbohydrates, alkaloids, tannins, flavonoids which were previously reported to be present in leaves of *M. oleifera* after preliminary phytochemical screening (Rastogi *et al.,* 2009).These compounds may pass through the different layers of the egg for the mortality action or inhibited the blastomere mitosis and blockage of the blastomeres segmentation of the egg that failed to embryonated.This mode of action is similar to that reported on compounds of albendazole (Ahmad *et al.*, 2011).

5. Conclusion and Recommendation

The findings of this study show that aqueous and ethanolic extracts of *Moringa oleifera* leafhave a potential anthelmintic activity on eggs in vitro which are comparable to a commercial Albendazole.The use of this plant for treatment against gastrointestinal nematodes especially on *Strongyle-type eggs* wasbetter beneficial with reduction of drug toxicity, drug resistance, and cost-effectiveness of our peasants. This miracle tree has strong bioactive compounds against parasites, microorganisms, and even some viruses with potential nutritional values to humans and animals. Therefore the ethnoveterinary of Ethiopia should focus on this plant to cultivate and expand its uses and the agricultural sector of the government should give attention to farmers for informing of such valuable plants to use for:-

- Medicinal purpose to their animals and human (Anticancer, anti-diabetes, and others)
- Food and ecological protections and prevention for land degradations
- For biofuels and animals feed beside to ovicidal and anthelmintic importances to come up of financial and drug-resistant of the parasites and some microorganism.

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