Thesis Ref. No_____

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTUER,

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY

IN VITRO ANTHELMINTIC ACTIVITY OF AQUEOUS AND METHANOLIC EXTRACTS OF *ALLIUM SATIVUM, ZINGIBER OFFICINALE AND RUTA GRAVEOLENS,* AGAINST *HAEMONCHUS CONTORTUS*

MVSc THESIS

BY

YODIT AYALEW

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JUNE 2022

BISHOFTU, ETHIOPIA

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A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Parasitology

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ADDIS ABABA UNIVERSITY

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DEPARTMENT OF VETERINARY PARASITOLOGY AND PATHOLOGY

As members of the examining board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by Yodit Ayalew entitled: ***In vitro*** **Anthelmintic Activity Of Aqueous And Methanolic Extracts of *Allium Sativum, Zingiber Officinale And Ruta Graveolens* Against *Haemonchus Contortus*** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of veterinary science in Veterinary Parasitology

Chairperson	**Signature**	**Date**
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I hereby certify that I have read the revised version of this thesis prepared under my direction and recommend that it be accepted as fulfilling the thesis/dissertation requirement.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an MVSc degree at Addis Ababa University, College of Veterinary Medicine and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Date of submission: _____

ACKNOWLEDGMENTS

Above all, Glory and praise to the almighty creator lord of the universe for his innumerable favor, blessing and benevolence up on me through all the way of my life.

I would like to express my sincere and heartfelt appreciation to my major advisor, Professor Getachew Terefe for his invaluable advice, encouragement, material support and devotion of his time on reading and correcting of this paper.

I am also grateful to my co-advisors Mr Takele Beyene (Associate Professor of Pharmacology) for his support and guidance and Professor Nigatu Kebede, for all technical, financial and material support.

This work was pertly supported by the Addis Ababa University graduate students fund and the Office of the Vice President for Research and Technology Transfer under the umbrella of the Thematic Research " Study towards control and prevention of major zoonotic diseases of veterinary and public health importance".

My deepest gratitude also goes to the College of Veterinary Medicine, the laboratory Assistants in the Parasitology Laboratory of CVMA, Mr Gebeyehu Alkadir and W/ro Tigist Gizachew and W/o Mahlet Arage of Aklilu Lemma Institute of Pathobiology.

I also acknowledge Bahir Dar University, School of Animal Science and Veterinary Medicine for sponsoring me to attend this postgraduate programme.

I have no words enough to appreciate my family members for their love and support throughout my ups and downs.

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LIST OF ABBREVATIONS

ALB	Albendazole
AL-IPB	Aklilu Lemma Institute of Pathobiology
AMIT	Adult motility inhibition test
CVMA	Collage of Veterinary Medicine and Agriculture
DMSO	Dimethyl sulfoxide
EHIT	Egg hatch inhibition test
EPG	Eggs per Gram of Feces
FEC	Fecal egg count
GIT	Gastrointestinal Tract
GINs	Gastrointestinal nematodes
IVM	Ivermectin
PBS	Phosphate buffered saline
LMIT	Larval motility inhibition test
WAAVP	World Association for the Advancement of Veterinary Parasitology
WHO	World Health Organization

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ABSTRACT

In vitro experimental trail were conducted from December 2021 to May 2022 to investigate the anthelmintic activities of aqueous and methanolic extracts of *Allium sativum (Garlic), Zingiber officinale (Ginger)* and *Ruta graveolens (Rue)* against *H. contortus*. The plants were purchased from local market from Bishoftu town. Methanol and aqueous extracts of the selected medicinal plants were prepared by maceration technique. Each plant was also subjected to qualitative phytochemical screening for the presence or absence of secondary metabolites such as alkaloids, phenolic compounds, flavonoids, saponins, tannis, triterpens and glycosides using standard procedures. Different graded concentration of the extracts were prepared and evaluated for *in vitro* anthelmintic effects using standard techniques of adult and larval motility inhibition test and egg hatch inhibition tests. For the adult motility inhibition test, 10 actively moving worms were placed into test tubes containing different concentration of plant extracts (100, 50, 25 and 12.5mg/ml). Similar concentrations were tested against larvae of *Haemonchus* but the number of worms was adjusted to 100 L3/well. The results were evaluated by counting the dead worms at an interval of three hours, for a total period of nine hours for adults and interval of six hours for 48 hours for the larvae. For the egg hatch inhibition test, the wells containing about 100eggs/ml were exposed to various concentration of the plant extracts (50, 25, 12.5 and 6.25mg/ml) and incubated at 27°C for 48 hours and evaluated based on the characteristics such as dead and hatched eggs. On the 3rd hour of observation, all higher concentrations (>25mg/ml) of both methanolic and aqueous extracts of *ginger* and *rue* have killed 100% of the adult worms. A similar level of efficacy for *garlic* was observed on the 9^{th} hour of exposure. Nine hours after treatment, only 20% of worms incubated with PBS or DMSO alone were found dead and the difference between treatment and control groups was significant ($P<0.001$). The effect of methanolic extract of the three plant species against adult *H. contortus* worms at all extract concentrations was equivalent to the efficacy of 5% Albendazole ($P>0.05$). Aqueous extracts had also similar effect except for *garlic* where significantly lower number of worms than the others including Albendazole ($P<0.05$) were killed.

Both Methanol and aqueous extract concentrations lower than 50mg/ml of all the three plants had significantly lower effect on *H. contortus* infective larvae compared to Ivermectin which has

killed 100% of the larvae. At higher concentrations, however, almost all extracts had greater than 80% efficacy and the difference was significant between treatment and both positive and negative control wells ($P < 0.005$).

Methanolic extract of *Ruta graveolens* had inhibited less than 60% of nematode egg hatchability at all concentrations tested whereas *ginger* extract had shown a concentration dependent increasing efficacy which goes beyond the effect of Albendazole although the difference was not statistically significant ($P > 0.05$). In a similar manner, aqueous extract of *rue* had much less ovicidal effect at all concentrations compared to the other two extracts and the positive control. In conclusion, the present study revealed the potential nematocidal and ovicidal effects of extracts from the three plant species. Further study is required to fractionate active compounds, support the finding by *in vivo* test and evaluate any toxic effect that may hamper the use of the products as potential anthelmintics.

Keywords*: Allium sativum; anthelmintic; extract; in vitro; Ruta graveolens; Zingiber officinale*

1. INTRODUCTION

Gastro-intestinal nematodes are one of the most important parasites that continue to pose a serious threat to the health and welfare of livestock. It is a diverse group of parasitic nematode which develops within the digestive tract (abomasum and intestine) of ruminants. It encompasses a wide range of nematode species, which mainly belong to the order *strongylidae,* superfamily *Trichostrongyloidea* significantly affecting the health of livestock (Mortensen *et al.,* 2003).

Worms in the genus Haemonchus are the most prevalent and severely pathogenic among the strongylids in livestock, particularly in small ruminants. It is a notorious parasite in ruminants due to its biotic potential and blood sucking ability (Tan *et al.,* 2014). Among the Haemonchus species, Haemonchus *contortus* is the most pathogenic and economically important abomasal parasite of small ruminants in the warm tropic and sub-tropical region of the world (Perry *et al.,* 2002). It is one of the most prolific strongylid nematode parasites which are capable of laying thousands of eggs each day, resulting in rapid larval pasture contamination resulting in the disease, Haemonchosis outbreaks. Haemonchosis can cause clinical symptoms such as anemia, followed by loss of appetite, lethargy, loss of weight, stunted growth, dehydration, edema and mortality as a consequence of the disease (Simpson, 2000, Angulo-cubillan *et al.,* 2007, Taylor *et al.,* 2007, Terefe *et al.,* 2007).

The most common control method of gastrointestinal nematodes (GIN) is largely based on repeated use of chemical anthelmintic drugs (Albendazole, Levamisole, Teramisole and Ivermectin). However, inappropriate and exclusive application of these drugs has contributed to the development of extensively drug-resistant parasites (Mideo *et al.,* 2013). This, in turn, increased risks of residues in the meat and milk of animals (WHO, 2006; Gasbarre *et al.,* 2001) and also environmental impact of drug residues in animal feces. Additionally, these synthetic agents are likely unaffordable and inaccessible or inadequately available to the resource poor farmers of developing countries (Hammond *et al.,* 1997).

These growing threats have long prompted researchers to focus on searching for new control approach methods like herbal remedies as alternative anthelmintic (Fajimi and Taiwo, 2005). Medicinal plants are considered as an alternative source of compounds that are biodegradable into non- toxic products and sustainable methods that are readily acceptable to rural farming communities. Also, it is an affluent resource of pharmacologically active ingredients which can provide an alternative to chemically synthesize drugs to which many infectious microorganisms have become resistant (Rasool, 2012).

Hence, evaluation of the activities of medicinal plants claimed for anthelmintic property is getting attention these days (Alawa *et al.,* 2003; Iqbal *et al.,* 2004; Pessoa *et al.,* 2002). The pharmacological studies and clinical trial going on in different part of the world have reported that a significant percentage of indigenous remedies of plant origin have shown promising biochemical activity and clinical effects.

For that reason, screening and proper evaluation of the claimed medicinal plants could offer the possible alternatives that may both be sustainable and environmentally acceptable (Githiori *et al.,* 2004).

The development of useful and widely used drugs derived from plant remedies such as Digoxin and Digitoxin, from Digitalis leaves; quinine from the cinchona bark; reserpine from *Rauwolfia serpentine*; morphine from *Papaver somniferum*; cocaine from *Erythroxzion coca* and the anti-cancer *Vincristiner* and *Viblastine* from *Cartharathus troseus* of Madagascar and again anti-cancer compound, bruceatin, from the Ethiopian plant, *Brucea antidysentrica*, just to name a few, are examples of the contributions of traditional pharmacopoeia (Endashaw, 2007).

The current study is designed to examine the anthelmintic activity of the common medicinal plants, *Ruta graveolens (Rue, Local Name: Tena adam), Allium sativum* (*Garlic)* and *Zingiber officinale* (*Ginger)* which grows in several parts of Ethiopia and elsewhere in the world. Extracts of these three plants are said to have different degrees of efficacy against gastrointestinal worms (Laudato and Capasso, 2013; Adeniji *et al.,* 2017). *Zingiber officinale* (*ginger*) powder was reported to reduce 92.6% FEC against *Strongyloides ransomi* of pigs (Kiambom *et al.,* 2021). According to Iqbal *et al*. (2006), both crude powder and crude

aqueous extract of the plant exhibited a dose- and a time-dependent anthelmintic effect on EPG when drenched to sheep. In a similar situation, *Allium sativum* was 100% effective *in vitro* when *H. contortus* worms were incubated with crude extracts for 6 hours (Iqbal *et al.*, 2001). Alcoholic extracts of *Ruta graveolens* L. leaves were examined for anthelmintic activity against Indian earthworms (*Pheretima posthuma*) as model where significant paralysis was observed (Pandey *et al.,* 2010). A closely related species, *Ruta chalepensis* extract had also shown anthelmintic efficacy *in vitro* (Ortu *et al.,* 2016).

Apart from these medicinal practices, the scientific information on the claimed efficacy of these plants is scant Thus; the importance of evaluating indigenous medicinal herbs for their degrees of potency against *H. contortous* becomes substantial.

Therefore, the objective of this study is; to investigate the *in vitro* anthelmintic potential of aqueous and methanolic extracts of *Allium sativum, Zingiber officinale* and *Ruta graveolens* purchased from local markets from Bishoftu town, on nematode eggs, adult and larvae of *Haemonchus contortus.*

Specific objectives are

- To assess the effect of time and extract concentration on the efficiency of the plant extracts on GIN eggs, *Haemonchus infective* larvae and adult.
- To compare the anthelmintic efficacy of crude extracts of the three plants on GIN egg, *H. contortus* infective larvae and adult parasites
- To assess the presence of different secondary metabolites in the extracts

2. LITERATURE REVIEW

2.1. Gastrointestinal nematodes

Gastrointestinal helminth infections are one of the most important diseases limiting livestock production. It is a ubiquitous parasitic agent that affects livestock particularly ruminants and are known to limit ruminant production in many areas and countries of the world (Adedipe *et al.,* 2014). Gastrointestinal helminthes cause significant economic losses of small ruminant enterprises through increased susceptibility of animals to other infections, morbidities, and mortalities, especially in heavily parasitized animals and in young animals. Among these, gastrointestinal nematodes are the leading causes of ill-health and production losses in livestock production worldwide.

Gastro intestinal nematodes are the most common and numerous parasites which develop within the digestive tract (abomasum, intestines) of ruminants. It is one of the major health problems in the world. According to Gillian *et al*. (2006), nematode infections affect the health of millions of people and animals, resulting significant economic loss in livestock farming. The consequences of nematode infection include: reduced feed intake and weight gain, reduced immunity, lower fertility, a reduction in animal products (meat, milk, hides and skin) and work capacity, treatment expenses and death in critical infections. Nematode parasites belonging to the order strongylida and superfamily *Trichostrongylidea* have a substantial impact on the health of livestock. Haemonchus *contortus* also known as the twisted stomach worm is the most common and severely pathogenic worms of these strongylid species in livestock, particularly in small ruminants (Perry *et al.,* 2002).

2.2. The Haemonchus Parasites

2.2.1. Parasite biology and epidemiology

The genus Haemonchus was first described in 1803 by Karl Rudolph (Soulsby, 1982) which is one of the most important endo-parasites of ruminants that belongs to the Phylum: Nematoda; Class: Secernentea; Order: Strongylida; Family: Trichostrongylidae; Genus: Haemonchus (Solulsby, 1986; Urquhart *et al.,* 1996). The genus Haemonchus is in the sub – family of Haemonchinae which is the largest ranging from 10-30 mm in length. It is a cylindrical

gastrointestinal nematode that is also known as red stomach worm, wire worm or the barber pole worm (selemon, 2018). It is a pathogenic hematophagus nematode that infects the abomasum of ruminant hosts (Vadlejch *et al.,* 2014). The genus Haemonchus consists of at least 12 species in which four main species in domestic ruminants, namely, H. *contortus* (in ovine and caprine), H. *placei* and *H. similis* (in bovine) and H. *longistipes* (in dromedary). H. *placei* and H. *contortus* are the most important species in cattle and shoats respectively (Lichtenfels *et al.,* 1994). These parasites are common blood feeders that causes anemia and reduced productivity and can lead to death in heavily infected animals (Khattak *et al.,* 2018). Haemonchosis caused by Haemonchus *contortus* species, is reported to be the most prevalent and highly pathogenic species in livestock, particularly in small ruminants (Terefe *et al.,* 2007). It is the major and economically the most important species in the tropical and subtropical regions of the world (Achi *et al.,* 2003; Terrill *et al.,* 2004).

Morphologically, in fresh specimens the worms can be easily seen due to their bright red color and considerable size and the most noticeable feature in females is the white egg filled uterus twisting spirally around the blood filled intestine, giving rise to the so called barber's pole effect (Gharbi *et al.,* 2013). In both sexes there is pair of wedge shaped cervical papillae in the esophageal region and a tiny lancet inside the buccal capsule used for piercing small blood vessels. The vulva is located about a quarter body length from the tail and may or may not be guarded by variously shaped cuticular inflations (vulvar flaps). The form of the vulvar flap may range from an extreme Lingui form shape to a knob shape or a complete absence (Linguiform, knobbed or smooth). The prevalence of these various vulvar flap configuration varies among species and subspecies (Kumsa *et al.,* 2007; Gharbi *et al.,* 2013).

It is indisputable that *H. contortus* is the most notorious parasite in ruminants due to its biotic potential and blood sucking ability (Tan *et al.,* 2014). Transmission can occur year round in favorable warm and humid climatic condition. L3 can survive in the pasture up to one year under these conditions. However in cooler climates, survival is usually weeks to a few months. Many external (environmental and internal (host) factor influence this pattern of development and thus the infection's epidemiology. Le- Jambre (1995) and Taylor *et al* (2007) have stated that temperature and humidity are the two most important components of the external environment.

2.2.2. Life cycle of Haemonchus

An understanding the lifecycle of nematode parasite is important for effective control programs. The majority of GIT nematodes have the same life cycle. Most of them are oviparous and the eggs are of a similar and very distinct type, with no immediate transmission of infection from one host to another. The life cycle of the genus Haemonchus (Figure 1) is direct and has pre-parasitic as well as parasitic stages (Roeber *et al.,* 2013). Adult female parasites live in the abomasum and produce eggs that are passed with in the feces. Each adult female parasite has a tremendous egg laying potential (5000-10000 eggs per day) (Emery *et al.,* 2016). Under favorable environmental conditions, especially in warm temperature and higher humidity, the eggs are excreted together with faeces then hatch and passes into first stage larvae (L1) which molts twice to become the 3rd stage infective larvae (L3). The L3 is attained in about 4 to 6 days after hatching. The L3 being infective to the host, are very active and motile moving up grass blades in the pasture where they are ingested by grazing animals. Then after ingestion, L3 exsheath in the rumen and move to the abomasum, where they penetrate the gastric epithelium into the gastric glands, then they molt and emerge back into the lumen as 4th stage larvae (L4). After that, they develop into immature adults (L5) for a short period of time and become mature adults soon after (Soulsby, 1982).

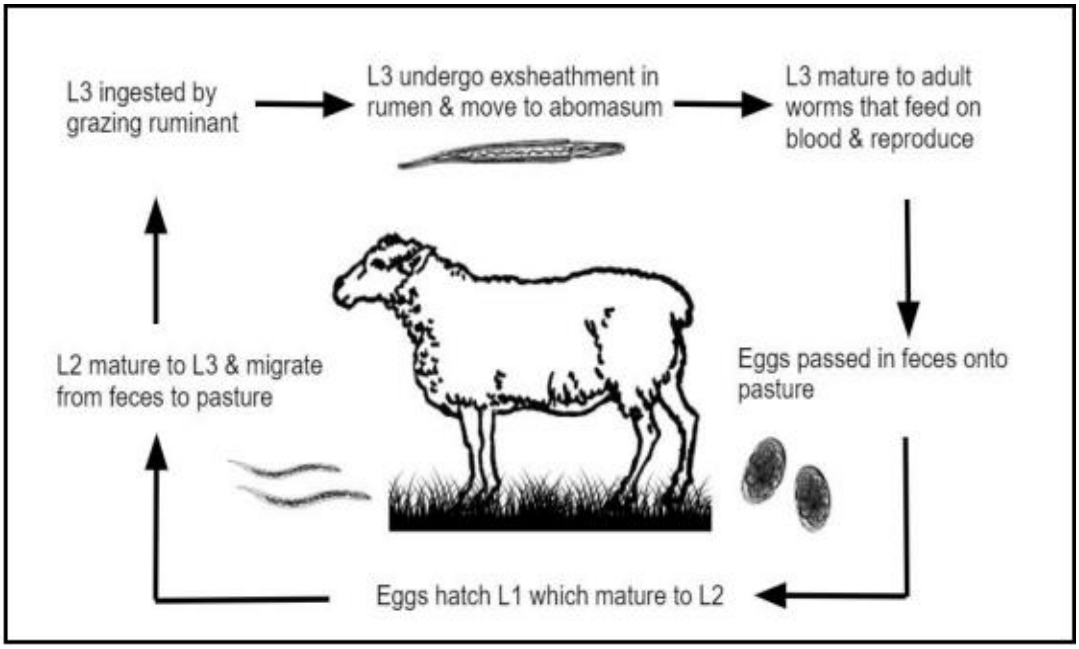

Figure 1.The life cycle of Haemonchus contortus in sheep

Source: (Roeber et al., 2013)

2.2.3. Pathogenesis and clinical signs of haemonchus infection

Helminthes are recognized as a major constraint to livestock production throughout the tropics and elsewhere in the world. The helminth parasites have been reported to cause loss in body weight reduced growth rate, delay maturity, general unthriftness and anemia and consequently huge economic losses. Losses from nematode parasitism are characterized by lower outputs of animal products (meat, milk, hides and skins), manure and traction, which all impact on the livelihood of small holder farmers (Gerold and Seip, 2006). The greatest losses associated with nematode parasite infections are sub-clinical, and economic assessments show that financial costs of internal parasitism are enormous. One exception is *Haemonchus contortus*, which is highly pathogenic nematode parasite of small ruminants that is capable of causing acute disease and high mortality in all classes of stock if left untreated (Cowan, 1999; Terefe *et al.*, 2007).

It is characterized by hemorrhagic anemia attributable to blood loss via the blood sucking activities of the worms in the abomasum. The mechanism of blood sucking involves the worms adhering to the abomasal mucosa and extruding its oral lancet to silt capillaries. Adults and fourth stage larvae consume blood from the split capillaries. They also secrete anticoagulants into the bleeding lesion, ensuring that the bleeding continues after the worm has moved away, and thus resulting hemorrhagic anemia. Through consumption and seepage from lesions, each worm removes about 0.05 ml of blood per day, thus a sheep with 5000 parasite may lose roughly 250 ml of blood every day. As a result; the degree of anemia is determined by the number of worms present in the abomasum (Urquhart *et al.,* 1996).

The pathogenic effect of H. *Contortus* results from the inability of the host to compensate for blood loss. The spectacular depression of hemoglobin level accompanied by weakness and death are the classical features of haemonchosis (Taylor *et al.,* 2007).

Haemonchosis may exhibit clinical signs such as anemia which is the most clinical sign, followed by lack of appetite, lethargy, loss of weight, dehydration, edema and death as a consequence of the disease (Simpson, 2000, Angulo-cubillan *et al.,* 2007, Taylor *et al.,* 2007, Terefe *et al.,* 2007).

2.3. Treatment and control of Haemonchosis

Nematode parasite control is critical for increasing livestock productivity and feed efficiency (Ketzis, 2003). Control of infection with these parasites is generally achieved by the use of commercial anthelmintics.

Commercial anthelmintics have been used for some decades throughout the world to minimize the losses caused by helminth infections (Baker *et al.,* 1994, Waller, 1999). However, the threats of anthelmintic resistance, risk of residue, availability and high cost especially to farmers of low income in developing countries have led to the notion that sustainable helminth control cannot be achieved with commercial anthelmintics alone. Therefore, today the strategy of helminth control has shifted to integrated control scheme involving grazing management, utilization of natural immunity together with anthelmintics for sustainable control of helminth parasites. In addition, other options like, biological control, vaccine and traditional medicinal plants are being examined in different parts of the world (Githiori *et al*., 2004).

2.3.1.Use of anthelmintics

Anthelmintics are the most commonly used method of helminth infection control Anthelmintics such as benzimidazoles, imidazothiazoles and macrocyclic lactones, have been utilized in veterinary medicine since the 1960s. Albendazole, thiabendazoles, mebendazole and febendazole are all benzimendazoles derivatives with broad spectrum of action against gastrointestinal nematodes (Roos, 1997). In addition to these classes, there are other drugs with specific anthelmintic activities. These classes are generally classified as narrow spectrum anthelmintic which includes organophosphates, substituted phenols and salicylanilides (Matrin *et al.,* 1997).

However, several reports have shown that the extensive use of these anthelmintics is constrained by growing development of resistance, high toxicity and environmental concern which has become a major practical issue in many African countries (Van Wyk and Mayhew, 2013) and elsewhere in the world (Le Jambre *et al.,* 1995; Eddi *et al.,* 1996).

2.3.2. *Anthelmintic Resistance*

Resistance to conventional anthelmintics is rapidly increasing in small ruminants, particularly in warm and humid climates, as a result of intensive and indiscriminate use of de-wormers, under dosing and a lack of anthelmintic class changes over time (Taylor *et al.,* 2007). Anthelmintic resistance refers to parasite heritable ability to withstand a normally effective dose of an anthelmintic. It is a decrease in the efficiency of an anthelmintic against a population of parasites that is generally susceptible to that specific drug (Sangster and Gill, 1999).

Resistance occurs when animals exposed to GIN show a decreased response towards an anthelmintic drug. Similarly, resistance results when certain populations of GIN possess a gene associated with resistance. If more than 5% of parasite survives a standard dose of a single anthelmintic drug, they are considered as resistant. The earliest reports of anthelmintic resistance involved nematode parasites of sheep and horses (Kaplan, 2004). Currently resistance has appeared in nematodes that affect many animal as well as humans. These include several phyla of helminths and cover all of the major chemical groups of anthelmintics (Sangster & Gill, 1999). Parasite resistance to benzimidazoles (i.e. albendazole, thiabendazole, and fenbendazole), imidazothiazoles (levamisole), and macrolides (ivermectin) has been reported in Australia, Africa, Europe, North America and South America; wherever animals are regularly treated with anthelmintics and investigations have been made (Suleiman *et al.*, 2005).

Helminth control in domestic animals is mainly based on chemical method in combination with grazing management. Widespread and intensive use of sometimes low quality anthelmintics are believed to be among the major factors contributing to resistance, leading to reduction in effectiveness of available anthelmintics (Githiori *et al.,* 2004). The main methods for delaying drug resistance are: infrequent use of anthelmintics, utilization of the most active anthelmintic compounds at the highest practical dose, yearly alternation of anthelmintics from different groups, management of pastures to avoid the buildup of resistant populations and surveillance of newly acquired stock (Kareru, 2008).

Coupled to the rampant resistance situation, high cost of modern anthelmintics and their unavailability has limited the effective control of helminthes by rural farmers. Chemical residues from anthelmintics are also reported to be toxic and can pose side effects to the administrator and the animal (Nalule *et al*., 2011).

Therefore, novel approaches to nematode parasite control are needed for livestock in the tropics and sub-tropics, to counteract the problem of anthelmintic resistance. Moreover, the cost of treatment and problem of accessibility has prompted the search for alternative medications. There is a need to rethink the use of anthelmintics, as well as develop novel approaches, which will lead to sustainable control of parasites. Ideally, this may entail an integrated approach, including biological control, reduced frequency of anthelmintic treatments, parasite vaccines, livestock breeds that are resistant to parasites, and the use of plant products/herbal medications with anti-parasitic properties (Waller, 1999).

2.4.Medicinal plants as alternative to chemotherapy

Since the time immemorial, our traditional system of medicine and folklore claiming that medicinal plants as a whole or their parts are being used in all types of disease successfully including anthelmintics (Jitendra *et al.,* 2010). Plant-based drugs are believed to be less toxic to the host and end-users. They are easily available, biodegradable, cheaper and eco-friendly. Moreover, the people have used them from generation to generation. Rural folk, tribes, ethnic groups and nomads have found several plants very effective for their day-to-day problems of health care. In livestock, medicinal plants are important in veterinary practice worldwide. Therefore, Phytomedicines in veterinary practice have great potential as alternative medicine (Kumar *et al.,* 2008).

2.4.1. History of medicinal plants

The history of ethno-botanical is almost as old as human civilization. It has been utilized to treat disease and mend injuries by humans since time immorial (Bevere, 1986). Medicinal herbs were known to be used by Chinese (100 B.C.), Romans (100 A.D.) and Indians for a variety of ailments. People all across the world relied on traditional (Folk) medicine for their day to day health care prior to the introduction of modern medicine (Lansk and Newaman,

2007). The indigenous people were able to recognize numerous plants, including their therapeutic potential, due to the presence of acquired knowledge over time.

Search for new and more effective remedies for controlling the disease of livestock has given rise to the study of plant based remedies. Plant based remedies plays an important role in animal production systems and livelihood development and often become the only available means for curing the ailments (Jabbar *et al.,* 2005). For centuries, traditional medicine in the form of herbal treatment holds a strong position in medical and veterinary care. It has been used as a source of medicinally useful components that are known to provide a rich source of botanical anthelmintics, antibacterial and insecticides.

Pharmacologically active compounds of plant origin can provide an alternative to chemically synthesized drugs to which many infectious microorganisms have become resistant (Akinpelu and Onakoya, 2006).

Medicinal plants nowadays serve as alternative remedies for both livestock and humans. In comparison with synthetic drugs, herbal medicines are economical, easy to consume and are locally available. The use of herbal remedies in the treatment of haemoncosis is potentially promising as they have been demonstrated to be potent anthelmintics (Lansk and Newaman, 2007; Jabbar *et al.*, 2005).

2.4.2. Some plant species with tested activity against gastro intestinal nematodes

Plants have provided the basis for traditional treatment for different types of diseases and still offer an enormous potential source of new chemotherapeutic agents (Jabbar *et al.,* 2005).

Different parts of plants have been used as anthelmintic agents based on the species of the plant; these are roots, bark, leaves and seeds. Table 1 shows some plant species with different parts that have been tested to have activity on haemonchus *contortus* and various species of nematode parasites of public and economic importance.

Table-1: Some medicinal plants and their parts used against various species of nematode parasites of animals and man

No	Plant species	Part	Extraction	Test parasite
1	*Mimusops elemgi*	Bark	Ethanol	*Ascaris galli*
2	*Pilostigma thonningi*	Stem bark	Methanol	*Haemoncus contortus*
3	*Calotropis procera*	Latex	Water	*Ostertagia Dictyocaulus*
4	*Carica papaya*	-	Water	*Ascaris lumbricoides*
5	*Buteneamonos ferna*	Seed	Methanol	*Caenorhabditis elegans*
6	*Acarcia nitotica*	Leaves	Chloroform	*Haemoncus contortus*
7	*Meliazoda Mexicana*	Fruit	Water Ethanol	*Ascadia galli*
8	*Anogeissus leiocarpus*	Leaves	Water	*Haemoncus contortus*
9	*Daniellia oliveri*	Stem bark	Water	*Haemoncus contortus*
10	*Fumaria paraviflora*	Whole plant	Water, Ethanol	*Trichuris*
11	*Rumex abyssinicu*	Leaf	Methanol	Endo-parasite
12	*Ruta chalepensis*	seed	Methanol	Helminth
13	*Leucas Martinicensis*	Root	Water	Endo-parasite
14	*Euphorbia abyssinica*	Young stem	Ethanol	*GIT* helminths
15	*Albezia anthelmintica*	Root bark	Ethanol	Helminths

Source: (Jabbar *et al.*, 2005).

2.4.3. Plants tested as anthelmintic in Ethiopia

Like many African countries, people in Ethiopia have also traditional methods of health care for both human and animals, which are widely practiced in a country side where modern veterinary service are limited.

Traditionally, plants have been used for the treatment of helminth parasites as a home remedy in the form of crude preparations or after random testing on parasitic problems of already prepared herbal medicines for other diseases (Kumsa and Hagos, 2020).

A number of plant species that have been used by traditional healers have been documented in Ethiopia Table 2 shows some plant species with different parts that have been tested to have activity on various species of gastro-intestinal parasites.

Table 2: List of some medicinal plants with tested anthelmintic activity in Ethiopia

No	Plant species	Parts used	Test parasite
1.	*Albizzia aummifera*	Leaf	Nematode
2.	*Croton macrastactiyum*	Bark	T. *saginata*
3.	*Cucumis spp*	Root leaf fruit	Helminths
4.	*Cucurbilo pepo*	seed	T. *saginata*
5.	*Allium sativum*	Bulbs	Adult parasite
6.	*Dodonea vicose*	Leaf	Nematode
7.	*Erebeliaschlaperi*	Fruit	T. *saginata*
8.	*Allium sativum*	Bulbs	Eggs
9.	*Glynus iotoides*	Seed	H. *contortus*
10.	*Hagenia abyssinica*	Flower	T. *saginata*
11.	*Jasminium martinicensis*	Leaf	Helminths
12.	*Linum usitatissimum*	Leaf	Helminth
13.	*Ruta chalepensis*	Seed	Helminths
14.	*S. oxyacantha*	Leaf	Helminths

15.	*C. Sativum*	Seed	Adult parasite
16.	*Zingiber officianale*	Rhizomes	Adult parasite

Source: (Kumsa and Hagos, 2020)

2.5. Plant extraction

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant from the inactive components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively complex mixture of metabolites, in liquid or semisolid state or in dry powder form that are intended for oral or external use (Handa, 2008).

The purpose of standardized extraction procedures for medicinal plant is to attain the therapeutically desired portion and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agents as in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites (Pandey and Tripathi, 2014).

A typical extraction process may contain steps of collection and authentication of plant material & drying, size reduction, extraction, filtration, concentration, drying & reconstitution (Handa, 2008).

The quality of an extract is influenced by several factors such as, plant parts used, solvent used for extraction, extraction procedure, and plant material: solvent ratio etc. The choice will also depend on the targeted compounds to be extracted (Pandey and Tripathi, 2014). While effect of extracted plant phytochemicals depends on the nature of the plant material, its origin, degree of processing, moisture content and particle size whereas the variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract depends upon type of extraction, time of extraction temperature, nature of solvent, solvent concentration and polarity (Ncube *et al.*, 2008).

For selection of solvents ‘like dissolves like’ principle is applicable. Thus polar solvents will extract out polar substances and non-polar material will be extracted out by non-polar solvents (Huie, 2002).

The variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract depend upon: type of extraction, length of the extraction period, temperature, nature of solvent, solvent concentration and polarity. Effect of extracted plant phytochemical depends on the nature of the plant material, its origin, degree of processing, moisture content and particle size (Pandey and Tripathi, 2014).

2.5.1. Methods of crude extraction

The mode of extraction depends on the texture and water content of the plant material being extracted and on the type of substance being isolated. Scientific analysis of plant compounds follows a logical pathway. Plants are collected either randomly or by a lead supplied by local healers. Initial screening for possible bioactivity typically begins by using aqueous or alcohol extractions which will be followed by various organic extraction methods. Since most of the identified components active against micro-organisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol (polar solvents) extraction (Cowan, 1999). Other polar solvents include, water, acetone and dimelthylsulfoxide (DMSO). The non-polar solvents are benzene, carbon tetrachloride, and petroleum ether. Polar solvents dissolve polar compounds, whereas nonpolar solvents dissolve nonpolar compounds.

Any part of the plant may contain active compounds. For alcoholic extraction, plant parts are dried, ground to a fine texture, and then soaked in methanol or ethanol for extended period. The slurry is then filtered, washed and dried. When water is used for extractions, plants are generally soaked in distilled water, bloated dry, made into slurry through blending and then strained or filtered. The filtrate can be centrifuged multiple times for clarification (Cowan, 1999).

In the case of solvent extraction technique, petroleum ether, ethanol, methanol, dichloromethane, chloroform etc. will replace water for extraction. For example, the powdered plant material is subjected to soxhlet extraction using first petroleum ether and then dichloromethane to remove lipophilic substances like plant chlorophyll. This is followed by

exhaustive extraction with acetone (actual solvent). Removal of the acetone under vacuum yielded a powder. The extract is placed in a vacuum oven at 40^0C before it is used for anti-parasitic testing. In soxhlet method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent (acetone, ether, alcohol etc.) in flask is heated and its vapors condense in a condenser. The condensed extract drips into the thimble containing the crude drug, and extracts it by contact (Cowan, 1999).

Other possible ways of solvent extraction are by using 10 volumes of *n*-hexane and 10 volumes of ethanol 95% by percolation. The extracts can further be fractionated on a Sephadex LH-20 column with methanol to give a number of chemical fractions. Percolation is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified solvent and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly (Cowan, 1999; Handa, 2008).

2.5.2. The use of plant extracts

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds.

Compounds isolated from plants have been grouped into chemical classes such as alkaloids, phenolic derivatives, terpenoids etc. (Hoet *et al.,* 2004). Phenolic extracts can be sub-classified into simple phenols, phenolic acids, quinines, flavonoids, tannins and coumarins etc (Table 3).

Table 3: Secondary metabolites found in medicinal plants

No	Chemical class	Subclass
1	PHENOLICS	Simple phenols
		Phenolic acids
		Quinines
		Flavonoids
		Flavones
		Flavonols
		Tannins
		Coumarins
2	TERPENOIDS, ESSENTIAL OILS	Capsaicin
3	ALKALOIDS	Berberine
		Piperine
4.	GLYCOSIDES	Peptidoglycans,
		glycolipids, glycoproteins

Source: (Awuchi, 2020).

2.6. Phytochemical screening

According to the WHO, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purpose, or which are precursors for chemo-pharmaceutical semi synthesis. Such plant contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as phytochemicals or phyto-constituents (Liu, 2004; Doughari *et al.,* 2009).

Phytochemicals are bioactive plant chemicals that have protective or disease preventive properties. They are natural compounds found in different plant parts and components. They are regarded as secondary metabolites that are naturally synthesized in all parts of the plant

body; bark, leaves, stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components (Tiwari *et al.,* 2011).

The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compounds (or active principles) which are more frequent in some plant parts than in others (Lahlou, 2004).

Phytochemicals have been recognized as the basis for traditional herbal medicine practiced in the past and currently in vogue in parts of the world (Lalitha and Jayanthi, 2012). In the search for phytochemicals that may be of benefit to the pharmaceutical industry, researchers sometimes follow leads provided by local healers in a region. Following such leads, plant parts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product (Das *et al.,* 2010).

2.7. Active secondary metabolites against Haemonchus

The unique biological activity of a plant can be identified by their phytochemical properties. Some phytochemicals have been demonstrated to have anthelmintic activity. Anthelmintic activity of plants is naturally attributed to their chemical content which may vary qualitatively and quantitatively in different parts of a plant (Tiwari *et al.,* 2011).

According to the analysis of the compounds in different plants, different secondary metabolites have been screened for their anthelmintic activities.

2. 7. 1. Tannins

Tannins are naturally occurring poly-phenolic compounds with high molecular weight. They are of two groups which are based on their structural types, hydrolysable tannins and condensed tannins. Condensed tannins are polymeric flavonoids which are most common in forage legumes, trees and shrubs (Min and Hart, 2003). Condensed tannins are relatively stable in the digestive tract of the animal and rarely have toxic effects (Githiori *et al.,* 2004).

Tannins have been reported to have anthelmintic properties. It have property that interfere with energy generation of worms by uncoupling oxidative phosphorylation or by binding to the free protein of gastrointestinal tract of the host animal or glycoprotein on the cuticles of the worms that leads to death (Patel *et al.*, 2010).

An *in vitro* study of anthelmintic activity of *Baliospermum montanum* by Mali and Wadekar, (2008) concluded that there was a possibility that tannins were responsible for the anthelmintic activity displayed against *Pheretima posthuma* and *Ascaridia galli*. In addition, Iqbal *et al.* (2007) had reported that the anthelmintic activity of condensed tannins against *H. contortus* has been evidenced by inhibition of egg hatching assay. Another study on the effect of condensed tannins in cassava by Netpana *et al.* (2001) concluded that feeding a supplement of cassava hay containing moderate levels of condensed tannins has an effect of reducing nematode egg counts in both cattle and buffaloes.

2.7.2 Saponins

The name saponin was derived from soapwort plant, whose root was traditionally used as soap. Saponins are glycosides of triterpenes, streroids and sometimes alkaloids and occur but - not exclusively in plants. Many of the plants contain little or no saponins while others, the triterpenoid saponins predominate. The oleanane-type of triterpene is the base structure found in the largest variety of medicinal plants (Ombasa *et al.*, 2012).
Saponins have been reported to have anthelmintic properties. It acts on the permeability of the cell membrane of the parasite causing vocalization and disintegration of teguments (Melzig *et al*., 2001).

Saponins were first reported to kill worms as early as 1962 using an extract from *Albizia anthelmintica*. Other studies have been done to characterize other plants believed to have anthelmintic effects. Ajayi *et al.,* (2008) reported through larval survival assay which revealed that *Aframomum danielli* have anthelminthic activity against gastrointestinal nematodes. Also a study reported by Deore and Khadabadi, (2010) prove the efficacy of *Chlorophitum borivilianum* root tuber against selected worms. From the results, they concluded that tubers could be used as anthelmintics and this further lead to confirmation that anthelmintic activity of *C. borivilianum* was due to presence of saponins.

2.7.3. Phenolic compounds

Some of the simplest bioactive photochemical consist of a single substituted phenolic ring. The site and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Githiori *et al*., 2004).

Phenolic compounds have anthelmintic activity that interfere with the energy generation mechanism by uncoupling the oxidative phosphorylation and also interfere with the glycoprotein of the cell surface of the parasite and causes death (Patel *et al.,* 2010).

Cowan (1999), have reported that Phenolic derivatives and tannins from the stem bark of *Mimusops elengi* has been found effective against *Ascaridia galli*. Also phenolic derivatives, Thymoquinone and dithymoquinone-cymene from *Nigella sativa* essential oil were found to kill earthworms and hookworms (Hoet *et al.,* 2004).

2.7.5. Alkaloids

Alkaloids are one of the main and largest components produced by plants, and they are metabolic by products that are derived from the amino acids. It consists of steroidal alkaloid and oligoglycosides which is responsible for anthelmintic effect. It acts as antioxidant, capable of reducing the nitrate generation which is useful for protein synthesis and suppresses the transfer of sucrose from the stomach to the small intestine, diminishing the support of glucose to the helminths. In addition, it acts on central nervous system and causes paralysis. The alkaloid pelletierine from the plant *Punica granatum* has been reported to act as potent anthelmintic against *H. contortus*. An *in vitro* study of anthelmintic activity of *M. oleifera* by Fahey (2005) concluded that there was a possibility that tannins, flavonoids, trierpenoids, saponins and alkaloids were responsible for the potent anthelmintic activity displayed against helminths and protozoans.

2.7.6. Flavonoids

Flavonoid consists of a large group of polyphenol compounds having a benzoyl- γ-pyron structure and is ubiquitously present in plants. They are synthesized by the phenylpropanoid

pathway. Available reports tend to show that secondary metabolites of a phenolic nature including flavonoids are responsible for the variety of pharmacological activities. It is hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection (Kumar and Pandey, 2013).

2.7.7. Terpenoids

Terpenoids are small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids show significant pharmacological activities such as anti-parasitic, anti-malarial, anti-inflammatory, anti-bacterial, anti-viral and anti-cancer activities (Boroushaki *et al.*, 2016).

As mentioned earlier, trepenoids were detected in most analysis plant species such as citrus fruit juice, *Hagenia abyssinica* leaves, Flax seeds, *Ruta chalepensis* leaves while in some plants its result depends on the type of solvents. Molan *et al.*, (2003) described that terpenoids have the ability to stop the adult motility and the consequent migration ability of ovine nematode parasites. It binds to surface molecules (proteins or sterols) inducing inhibition of the protein expression, and/or lyses of the cell.

2.8. Studies on the whole plant

The variety of methodologies used for this purpose includes the provision of fresh, conserved or dried plants or plant parts to parasitized animals. For example, the consumption of leaves of wormwood in the form of powder (*Artemisia brevifolia*), one of the bitterest of plants, has been tested in a controlled study for its anthelmintic activity. Iqbal *et al*. (2004) demonstrated that the consumption of the whole plant resulted in a 62% reduction of *H. contortus* egg counts. The consumption of fagara leaves (*Zanthoxylum zanthoxyloides*), a native tree from Africa, believed to have anti-parasitic activity, resulted in reduced egg excretion by the same nematode in sheep, when consumed regularly in small amounts (Hounzangbe-Adote *et al.*, 2005). However, not in all cases the evidence on the anti-parasitic properties of plants is consistent with the expectations arising from traditional views. When leaves of the neem tree (*Azadirachta indica*) were offered to parasitized sheep, either fresh or dried, no anthelmintic effect was recorded against *H. contortus* by Githiori *et al*. (2004) and; Costa *et al*. (2008)

whereas, Tabassam *et al*. (2008) reported the effectiveness of neem in reducing worm numbers of the same nematode in small ruminants suggesting the existence of factors affecting the efficacy of these plants.

The interpretation of the observed inconsistencies in the activity of medicinal plants when offered to parasitized animals, either as a supplement or single food, is not straightforward. In cases where evidence is anecdotal, one part of the problem seems to be the misinterpretation of facts in various communities, often due to the lack of scientific knowledge. For example, traditional healers are not always familiar with the parasite species that are most pathogenic for livestock. Thus, in some cases, plants may have been mistakenly included in lists with those reported with anthelmintic properties and these mistakes may be justified when controlled experimentation is performed. In other cases, the inconsistencies observed might be related to methodological limitations while testing the anthelmintic properties of medicinal plants. The majority of ethno veterinary reports originate from ruminants, as the main livestock species that generate income in poor countries (Satrija *et al*., 2001). Consequently, when the anti-parasitic activity of such plants is tested in rodent models, for example in the form of dried plant, part of the reported variation may be due to the physiological difference between ruminant and non-ruminant animals (Athanasiadou *et al.,* 2007).

2.9.Testing for the anthelmintic activity of medicinal plant

In vitro and *in vivo* studies are usually done to assess anthelmintic activities of the medicinal plants. *In vivo* test, involves animal models such as mice, rats, ruminant animals which are infected with helminths. The infected animals are treated with herbal extract followed by monitoring helminths eggs in the animal faeces over time after the drug is administered. Reduction of faecal egg counts with time is an indication of efficacy of the plant extract (Githiori *et al.,* 2004).

In vitro studies involve culturing helminth egg and larvae from animal faeces either at room temperature or in an incubator and monitoring of death of adult worms. The effects of herbal products are performed by egg hatch inhibition, larval mortality and adult worm mortality assays. The percentage of egg hatch inhibition and mortality of the larvae are then determined.

For adult worm mortality assay, the time of the dead adult worm is recorded (Iqbal *et al.*, 2007).

2.9.1. In vitro efficacy studies of medicinal extracts

Scientific validation of anthelmintic activity has mainly based on *in vitro* studies. Many of the *in vitro* investigations on anthelmintic activity of plants, their oils, or extracts have been based on their toxic effects on the earthworm, *Pheritima posthum* or on the free-living nematode, *Caenorhabditis elegans*. Some workers have also used hookworms and tapeworms for the evaluation of *in vitro* anthelmintic activity of different plant materials (Akhtar *et al.,* 2000). Similarly, *in vitro* studies have been conducted to see the effects of plant extracts on the parasite egg hatchability, motility and survival of larval and adult worms of animals and man including *Haemonchus contortus* of sheep and goats, *Ascaridia galli* of poultry and *Ascaris lumbricoides* of man (Lateef *et al.,* 2005). The assumption made in these *in vitro* studies is that the intensity and types of activity observed against the model nematodes will be similar in infected livestock and man. *In vitro* tests are valuable for initial screening and to establish biologically realistic drug concentrations for further animal testing (Pessoa *et al.,* 2002).

The method includes keeping eggs, larvae or adult worms in physiological saline, addition of the test plant extract at different concentrations including known anthelmintic drug for comparison, and observation of the desired effect: hatchability, mobility, physical damage or death.

Reports showed remarkable vermicidal activity of *Calotropain* (proteolytic enzyme isolated from the latex of *Calotropis procera*) and Bromelain (an enzyme obtained as a by-product from pineapple industry) against *Oesophagostomum columbianum* and *Bunostomum trigonocephalum* of sheep origin compared to phenothiazine (Akhtar *et al.,* 2000).

2.9.2. *In vivo* method

In vivo studies are more reliable than *in vitro* methods although cost of large scale screening of plant extracts is probably inhibitory. It requires parasitized hosts (naturally or experimentally). *In vivo* trials have been conducted for the evaluation of anthelmintic activity of various substances of plant origin. These included expulsion of worms from their hosts or reduction in

the number of eggs per gram of feces (EPG) passed by the infected hosts compared with commercial anthelmintic treated animals (Akhta *et al.,* 2000). The method includes grouping infected animals into plant extract treated and commercial anthelmintic treated groups with another group remaining as non-treated serving as negative control.

2.9.3. Haemonchus contortus as a model system in anthelmintic discovery

The effects of several plant extracts on *H. contortus* and other important gastrointestinal nematodes using egg hatch and larval Mortality tests have been examined by many authors (Aroche *et al.,* 2008; Gabino *et al.,* 2010).

Haemonchus contortus has a very high propensity to develop resistance to anthelmintics and is the parasite species which has developed resistance most rapidly and in which resistance is most widespread (Kaplan, 2004). Partly because of this, and partly because of its economic importance and experimental tractability, it is the helminth species for which we have the greatest understanding of mechanisms, genetics, diagnosis and management of anthelmintic resistance (Gilleard, 2006). It is also one of the parasites for which we understand most about the modes of action of anthelmintics (Saunders *et al.,* 2013).

Consequently, it already provides a framework for much of the anthelmintic resistance and drug discovery research occurring in other parasite species. *H. contortus* has also been an extremely important organism in anthelmintic discovery research within the pharmaceutical industry for many years. An excellent recent example of the value of *H. contortus* for drug discovery and mode-of-action studies has resulted in the development of a new anthelmintic monepantel (Kaminsky *et al.,* 2008).

2.10. Description of plants used in the current study

Selection of plants was based on the literature survey on the traditional uses of the plants in Ethiopia and other parts of the world. Also easy availability of the plant material in the required amount and existence of studies researching their biological activity on nematodes was also considered as criteria. Brief descriptions of these three plants evaluated in the current study are given below**.**

2.10.1. *Ruta graveolens* (*Rue*)

Ruta graveolens is commonly known as *rue* (local name: *Tena adam*) which belongs to the plant family *Rutaceae* and order of *Sapindales* (Raghav *et al.*, 2006). It is a woody, strongly aromatic, perennial herb, native to the southern Europe and northern Africa. It is also very abundant in Brazil and other tropical countries (Stashenko *et al.,* 2000). Leaves are dissected innately into oblong or spoon-shaped segments as shown in figure-2, fleshy and usually covered with a powdery bloom.

Whole plants are largely used in traditional system of medicine as antiseptic, stimulant, abortifacient, anti-rheumatic, anti-helminthic, anti-colic, anti-spasmodic and anti-epileptic (Ahmad *et al.,* 2010). According to the literature, species of the genus *Ruta* have presented several biological activities, such as antioxidant, antifungal, phytotoxic, antidepressant, abortifacient and anti-inflammatory (de feo *et al.*, 2002, Kabouche *et al.,* 2003, Meepagala *et al.,* 2005, Raghav *et al.,* 2006).

Ethanolic, methanolic, chloroform and water stem extracts showed potent antimicrobial activity while its alcoholic leaves extracts showed potent anthelmintic, *in vitro* antioxidant and α-amylase inhibitory activity (De Freitas *et al.,* 2005). In addition, Alcoholic extracts of *Ruta graveolens* L. leaves were examined for anthelmintic activity against Indian earthworms (*Pheretima posthuma*) as model where significant paralysis was observed (Pandey *et al.,* 2010). A closely related species, *Ruta chalepensis* extract had also shown anthelmintic efficacy *in vitro.*

Phytochemical studies have attributed these biological activities to the presence of metabolites such as coumarins, alkaloids, flavonoids, ketones, terpenoids and others (Stashenko *et al.,* 2000, De Freitas *et al.,* 2005, Ahmad *et al.,* 2010).

Figure 2: Leaves of *Ruta graveolens*

(Source: Asgarpanah and Khoshkam, 2012)

2.10.2. *Zingiber officinale*

Zingiber officinale, commonly called *ginger* which belongs to *Zingiberaceae* family, is a perennial plant with narrow, bright green, grass-like leaves about 1 m tall long and 2-3 cm broad and yellowish green flowers with purple markings (Grant, 2000). *Ginger* is cultivated in the tropics for its edible rhizome at approximately 10 months of age. Rhizomes are aromatic, thick lobed, knobby and fleshy, covered in ring-like scars (Figure-3) with the root stocks serving a variety of purposes, including folk medicine and as a spice (Rahuman *et al.,* 2008). *Z. officinale* is considered to be the universal medicine in different countries. Traditionally, the rhizomes of *Z. officinale* are used for abdominal bloating, coughing, vomiting, diarrhea and rheumatism. It also stimulates digestion and keeps the intestinal muscles toned (Iqbal *et al.*, 2001).

Scientific reports have shown that extracts from rhizomes of *Z. officinale* have several pharmacological effects such as antimicrobial activity, anti-cancer activity, antioxidant activity, anti-diabetic activity, anti-hyperlipidimic and hepatic anti-cancer effect, nephron-protective activity, hepato-protective activity, larvicidal activity, analgesic activity, anti-inflammatory activity and immune-modulatory activities. Also, some *in vitro* as well as *in vivo* studies have been carried out to proof the anthelmintic efficacy of *Z. officinale* rhizome on various parasite species (Moazeni and Khademolhoseini, 2016). According to Iqbal *et al*.

(2006), both crude powder and crude aqueous extract of the plant exhibited a dose- and a time-dependent anthelmintic effect on EPG when drenched to sheep. In addition, its powder was reported to reduce 92.6% FEC against *Strongyloides ransomi* of pigs (Kiambom *et al.*, 2021). Moreover, its efficacy as a molluscicide against *Biomphalaria alexandrina*, the snail intermediate host of *S. mansoni* was reported by Omran *et al*. (2007).

The phytochemical screening indicated the presence of chemicals such as essential oils, phenolic compounds, flavonoids, carbohydrates, proteins, alkaloids, glycosides, saponins, steroids, terpenoids and tannins. The oil from *ginger* is believed to be very medicinal. The major active ingredients in *ginger* oil are reported to be the sesquiterpenes, which include bisapolene, zingiberol and zingiberene (Lakshmi and Sudhakar, 2010).

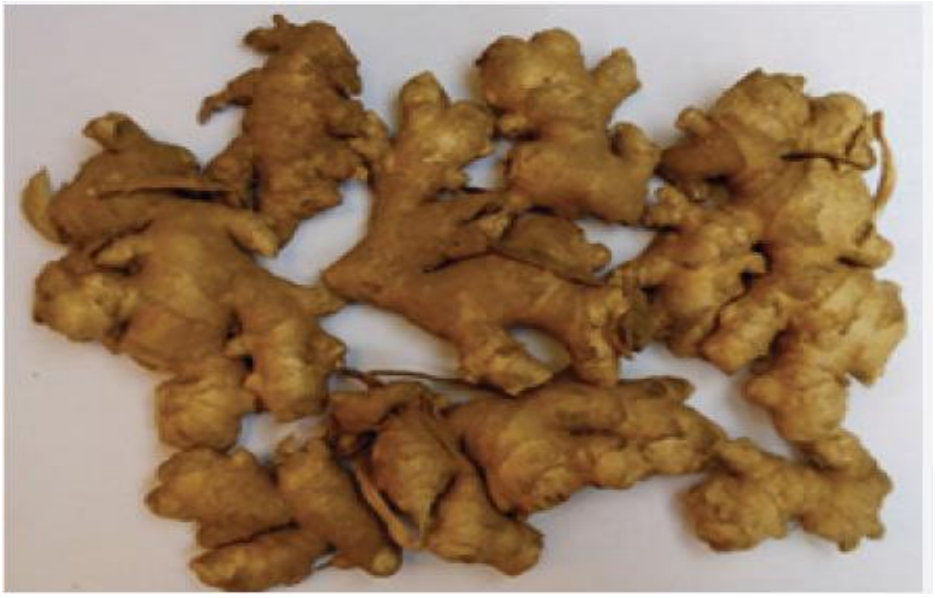

Figure 3-The rhizomes of *Z. officinale*
(Source: Imo. and Za'aku, 2019)

2.10.3. Allium sativum

Allium sativum commonly known as *Garlic* is among the oldest of all cultivated medicinal plants. It has been used as a spice, food and folklore medicine for many years, and has been widely researched as a medicinal plant (Imo and Za'aku, 2019). It is a member of the *Liliaceae* family and a species in the onion genus, *Allium.* It is one of the most popular bulbs used worldwide to reduce various risk factors associated with several diseases (Duke, 2002). In herbal medicine, it is used to prevent hypertension, wound healing potential and has

antioxidant activity (Hughes and Lawson, 1991). Traditionally in Ethiopia, the fresh bulbs are cooked and eaten to treat asthma, colds, coughs, and to control worms. Beside these, it also possesses some anthelmintic activity against roundworms and flukes (Palacio-Landín *et al.*, 2015).

Extracts from bulb of *A. sativum* has been reported several pharmacological effects such as anti-bacterial, anti-viral, and anti-fungal, parasiticide, amoebicide, larvicide and immune-stimulant activity (Duke, 2002). Essential oil of these plants can also be used as alternative or supplement to the current anthelmintics therapies.

The alcoholic extract of bulb of *A. sativum* has also shown moderate *in vitro* anthelmintic activity against human *Ascaris lumbricoldes* (Anthony *et al.*, 2005). In addition, according to Iqbal *et al*. (2006), both crude powder and crude aqueous extract of the plant exhibited a dose- and a time-dependent anthelmintic effect on EPG when drenched to sheep. In a similar situation, *Allium sativum* was 100% effective *in vitro* when *H. contortus* worms were incubated with crude extracts for 6 hours.

Also *A. sativum* has been reported to be effective in the exposure of dysentery and also act as vermifuge. Oil of *A. sativum* has also been reported to possess anthelmintic activity and discards all injurious parasites in the intestine (Perry *et al*., 2002).

Some bio-compounds obtained from *garlic* are allicin, allinin, ajoene, and diallylsulfide that are sulphured compounds. It is the best known source of selenium. The sulfur compound allicin, produced by crushing or chewing fresh *garlic*, in turn produces other sulfur compounds: ajoene, allyl sulfides, and vinyldithiins (Koch and Lawson, 1996).

Figure 4-Cloves of *A. sativum*

(Source: Imo. and Za'aku, 2019)

Table 4: Plant materials used for the study evaluated for their anthelmintic activity

Botanical name Family	**Family**	**Parts used**	**Common Names**
Allium sativum (a bulbous herb)	*Liliaceae*	*Bulb*	*Garlick, Lehsan, Lassan, Lasun, Lashan*
Zingiber officinale (a rhizomatous herb)	*Zingiberaceae*	*Rhizomes*	*Ginger, Adrak, Sonth (dried), Ada*
Ruta graveolens	*Rutaceae*	*Leaf*	*Rue, Tena adam*

3. MATERIALS AND METHODS

3.1.The study area

The present experimental study was conducted at Addis Ababa University, Collage of Veterinary Medicine and Agriculture (CVMA) while qualitative phytochemical screenings were done at Aklilu Lemma Institute of Pathobiology (AL-IPB), Addis Ababa University (AAU).

3.2. Study design

The study design was experimental research design which was conducted from December 2021 to May 2022 to evaluate the *in vitro* anthelmintic activities of aqueous and methanolic extracts of *Cloves* of *Allium sativum, rhizomes of Zingiber officinale* and leaves of *Ruta graveolens* against *Haemonchus contortus*. Extracts were tested with different concentrations and different exposure time intervals on egg, larvae and adult parasite.

3.3. Collection of Plant Samples and extraction methods

Cloves of *Allium sativum* (*Garlic*), rhizomes of *Zingiber officinale* (*Ginger*) and Fresh leaves of *Ruta graveolens* (*Rue*), were purchased from local market, in Bishoftu town and were transported to the Veterinary Parasitology laboratory. The materials were thoroughly cleaned and carefully inspected. Those with gross lesions or damage were discarded and the intact ones were washed gently with tap water to remove dirt and soil and were left to air dry under shade for 10 days. Rhizomes of *ginger* and peeled cloves of *garlic* were chopped into smaller pieces to facilitate drying. The dried materials were then ground and coarsely powdered using an electric grinder. The powder was sieved, weighed, labeled and conserved for extraction. The procedure was conducted according to Eguale *et al.* (2007).

3.3.1. Preparation of Aqueous extracts

The Aqueous extracts were prepared by dissolving 200g of dried and powdered plant material in 1000ml of distilled water in a conical flask. The material was allowed to macerate for 72h at room temperature with frequent agitation. The mixture was later strained using a muslin cloth and filtered using a Whatman No.1 filter paper. The filtrate was collected in a flask and later concentrated using rotary evaporator (Rota vapor: BUCHI R-200, Labor-technik Switzerland) at 100^{o}C under reduced pressure (700mmHg) and rotation speed of 20rpm followed by drying in an incubator at 40°C in order to obtain dry extract. After complete solvent evaporation, the extract was scraped off, weighted and kept in labeled bottles at 4^{o}c until used.

3.3.2. Preparation of Methanol extract

Methanol extracts were prepared by soaking each dried and powdered plant material (200g) in a conical flask to which 1000ml of 70% methanol solvent was added. The mixture was allowed to macerate for 72h at room temperature with frequent agitation after which it was strained using a muslin cloth and filtered using a Whatman No.1 filter paper. The filtrate was collected in a flask and later concentrated using rotary evaporator (Rota vapor: BUCHI R-200, Labor-technik, Switzerland) at 60^{o}C under reduced pressure (700mmHg) and rotation speed of 20rpm followed by drying in an incubator at 40°C in order to obtain dry extract. After complete solvent evaporation, the extract was scraped off, weighted and kept in labeled bottles at 4^{o}c until used. The extract procedures were prepared following the methods of Tabassam *et al*., (2008).

3.4. Phytochemical Screening

All Aqueous and methanol extracts were screened for the presence and absence of different phytochemicals/secondary metabolites. Standard screening tests using conventional protocol were conducted to identify the constituents as described by various authors (Evans, 1996; Briggs, 1970; Dermarderosian and Liberti, 1988; Rafauf, 1970). Accordingly, presence or absence of phenols, Tannins, Sterols, Saponins; Glycosides; flavonoids and alkaloids were determined (annex I). Depending on the type of test, positive results were determined by the

presence of color changes, precipitations or foam formation after reaction with different reagents.

3.5. Preparation of working Concentrations of extracts

The stock solutions of the crude extracts obtained from test plants were prepared by dissolving the crude aqueous extract in distilled water and the methanol extract in 5% DMSO. The aqueous and methanol extracts of the three plants were tested in triplicates by using 96 well micro titer plates for the larval motility test and egg hatch test while for adult motility tests; 10ml test tubes were used. Stock solutions of the extracts were first prepared and then further diluted serially to obtain effective concentration.

3.6. Reference Drug

The anthelmintic drugs, Albendazole and Ivermectin (Table 5) as positive control agents were bought from local retail markets. Ivermectin (1% solution) was used as positive control for larval motility assay at a concentration of 0.5mg/ml (Delfin, *et al.*, 2017), while Albendazole was used for egg hatch inhibition and adult motility assays at concentrations of 5mg/ml as used by Sileshi *et al.(*2012).

Table 5: Details of the anthelmintic drugs used in the vitro experiment

Anthelmintic drugs used	**Trade name**	***In vitro* test conducted**	**Manufacture countries**
Albendazole	Albenda	AMIT and EHIT	Chengdu Qiankun veterinary Pharmacy, Sichuan province, China (Albenda-qk 300mg)
Ivermectin	Tecmectin (1%)	LMIT	Chengdu Qiankun veterinary Pharmacy, Sichuan province, China

3.7.Parasite collection and egg preparation

3.7.1. Adult Haemonchus contortus

Adult parasites of *H. contortus* were collected from the abomasum of sheep slaughtered in ELFORA abattoir in Bishoftu town. Abomasa were checked and detached soon after evisceration and transported to the Parasitology laboratory in College of Veterinary Medicine and Agriculture of the Addis Ababa University. Each abomasum was opened along its greater curvature and its content was emptied into plastic bucket by flushing with water. Then, the parasites were recovered by passing the content through a sieve of 100 micrometer diameter wire mesh and were picked with a wire loop, washed and suspended in phosphate buffered saline (PBS) until the *in vitro* evaluation test began (within one to two hours of collection).

3.7.2. Culturing and recovery of Haemonchus contortus infective larvae

To obtain infective larvae, Adult female *H. Contortus* parasites were obtained as described above and separated from male parasites based on their morphological characteristics. As much as female parasites as possible were pooled and crushed using mortar and pestle to liberate the eggs. Then, the eggs were seeded on glass jar containing sterilized sheep faeces for 10 days at room temperature according to the method previously described (Mcintyre *et al.*, 2018). Infective larvae (L3) were harvested by filling the jar containing fecal culture with warm water and inversing upside down by covering the top with Petri dish followed by addition of some warm water to the petridish in to which the larvae migrate. After one hour, larvae containing water was pipetted and collected in a universal bottle. The viability was checked and larvae were quantified (concentration per milliliter) under stereomicroscope.

3.7.3. Collection of eggs of Gastro-intestinal nematodes

The eggs of nematodes were isolated from fecal samples collected from intestines of slaughtered sheep (ELFORA abattoir) harboring Haemonchus worms in their abomasa. Hence, it is possible that eggs of other gastrointestinal worms such as Trichostrongylys could be

present in the mixture. Eggs were concentrated using fecal flotation technique. Briefly, the fecal pellets were crushed using pestle and mortal, Water was slowly added to the feces and the pellets stirred until a relatively uniform homogenate liquid suspension was obtained. The suspensions were filtered through sieve and centrifuged for 2 min at 2000rpm and the supernatant decanted. Saturated sodium chloride floatation fluid was added to the test tube until formation of meniscus and then cover slip were put over the meniscus for 10 min. The cover slips were carefully removed and gently flashed with distilled water into a test tube to collect the eggs (Coles *et al.,* 1992). Then after collection, it was agitated and 100µL was transferred into petri dish marked with grid in order to estimate the concentrations of eggs in aliquots in 1ml before diluting it to the required concentration for use in the intended test (Viviane *et al.*,2017).

3.8. *In vitro* anthelmintic activity of the extracts

For evaluation of anthelmintic activity of the aqueous and methanol extract under *in vitro* conditions, the egg hatch inhibition test (EHIT), the larval motility inhibition test (LMIT) and the adult motility inhibition test (AMIT) techniques were adopted. It was conducted by applying the guideline of World Association for the Advancement of Veterinary Parasitology (WAAVP) adopted by Coles *et al*. (1992), Iqbal *et al*. (2004) and Eguale *et al*. (2007) with certain modification.

3.8.1. Adult worm motility inhibition Test (AMIT)

For the evaluation of anthelmintic activity of the aqueous and methanolic extract under *in vitro* conditions against adult *H. contortus,* the worm motility inhibition assay was adopted as described by Hounzangbe-Adote *et al.* (2005).

Ten actively moving worms were placed in to test tubes containing 100, 50, 25 and 12.5mg/ml of aqueous and methanolic extracts of each plants diluted in distilled water (Azra *et al*., 2019) and 5% dimethyl sulfa oxide (DMSO) respectively and Albendazole, a well-known anthelmintic, Albendazole-300mg, at a concentration of 5 mg/ml according to guidelines of (Sileshi *et al.,* 2012) was used as the reference drug (positive control). DMSO (5%) and PBS

were the negative control groups. Three replications per each treatment concentration were employed.

Inhibition of worm motility was taken as an indication anthelmintic activity. Motility of the worms was observed on different time intervals at 0, 3, 6, and 9hr. post exposure intervals and on every observation motile worms were counted.

After 9hr post exposure, the extracts and Albendazole were washed away and parasites re-suspended in Luke warm PBS for 30 min to test the revival of the worm motility. Then, the number of motile (alive) worms were counted under dissecting microscope and recorded for each concentration. Death of worms was ascertained by their straight flat appearance with absence of motility at the head and tail regions of the body for 5–6s. A mortality index was calculated as the number of dead worms divided by the total number of worms.

3.8.2. Egg Hatch Inhibition Test (EHIT)

The ability of the extract to inhibit egg hatching was conducted according to the procedure described by Coles *et al.* (1992). For each extract, a stock solution of 100 mg/ml was taken as the initial concentrations and serial dilution was made to a concentration of 12.5, 25, 50 and 100 mg/ml (Kanojiya *et al*., 2015). Then, the egg suspension, with a concentration of approximately 100eggs/0.1ml of distilled water was pipetted into each well (in a 96-well polystyrene plate). To each of the test wells, 100 µL of each plant aqueous and methanol extract was added to a final volume of 200 µL per well. Since eggs are in 100µl of distilled water, the final test concentration has become 6.25, 12.5, 25 and 50mg/ml. Similarly, 100 µL of Albendazole 300 mg at a concentration of 5 mg/ml was dissolved in distilled water which was used as a positive control while 100 µL of PBS or 5% DMSO was added to untreated eggs as negative control as described by (Costa *et al.,* 2008). Three replicas were made for each concentration. Finally, the wells were covered in order to prevent drying out and incubated at 27°C for 48 hours. After 48 hours, a drop of Lugol's iodine was added to both fix and stain the egg and larvae. Then, 100µL of agitated sample was transferred into a Petri dish marked with grid (Viviane *et al.,*2017) and, all eggs in each well were counted under stereomicroscope and grouped into non-embryonated/dead, larvated and hatched (considered together as hatched larvae)

Egg hatch inhibition was calculated as

$$EHI = \frac{\text{Number of non-embryonated or dead egg}}{\text{Hatched larvae + Non- embryonated eggs}} \times 100$$

3.8.3. Larval Mortality inhibition Test (LMIT)

Larval mortality assay were performed according to Wabo *et al.*, (2006). For each extract, a stock solution of 200mg/ml was taken as the initial concentrations and serial dilution was made to a concentration of 25, 50, 100 and 200 mg/ml. Then, the larvae suspension, with a concentration of approximately 100 larvae/0.1ml of distilled water was pipetted into each well (in a 96-well polystyrene plate). To each of the test wells, 100 µL of each plant aqueous and methanol extract was added to a final volume of 200 µL per well. Since larvae are in 100µl of distilled water, the final effective concentration was 12.5, 25, 50 and 100mg/ml. Similarly, 100 µL of Ivermectin (1%) at a concentration of 0.5 mg/ml was used as a positive control as described by (Delfin, *et al.*, 2017), while 100 µL of either PBS or DMSO was added to untreated larvae as negative control. Three replicas were made for each concentration and control. Finally, the wells were covered and incubated at 27°C for 48 hours. Inhibition of larval motility was taken as an indication of anthelmintic activity (Larval killing ability). Mortality was monitored at 0, 6, 12, 24 and 48h intervals. All larvae in each well were counted under a stereo-microscope and classified into motile and non-motile/dead. The results were expressed as

$$LMI = \frac{\text{Number of non-motile larvae}}{\text{Total number of larvae}} \times 100$$

4. STATISTICAL ANALYSIS

The data of this study was organized, edited and entered on excel word work sheet, the results of egg hatch inhibition, mortality of adult and larval stage at different concentrations were converted into percentage and comparison of different mortality rates were made using Chi-square test, calculated by Med Cal online tool (https://www.medcalc.org/cal/comparsion_of proportions. php) and the results regarded as significant at $P< 0.05$.

The crude methanolic and aqueous extracts were subjected to qualitative phytochemical analysis using standard methods.

5. ETHICAL APPROVAL

All experimental procedures were approved by the ethics committee of Addis Ababa University, Collage of veterinary medicine and Agriculture (Ref No. VM/ERC/07/02/14/2022)

6. RESULTS

6.1. Phytochemical screening

Phytochemical screening of the aqueous and methanol extracts of *Ruta graveolens, Allium sativum* and Z*ingiber officinale* had revealed the presence of different secondary metabolites. The major secondary metabolites detected in all the extract type are listed in table 6.

Table 6: Phytochemical constitutes of the investigated plant extracts

Secondary metabolites	Plants used in the study					
	Zingiber officinale		***Ruta graveolens***		***Allium sativum***	
	M. E	A. E	M. E	A. E	M. E	A. E
Saponins	+++	++	+	+	+	+++
Alkaloids	+	+	+	-	+	++
Flavonoids	+	-	-	++	-	+
Glycoside	+	+	+	+	++	+
Steroids	-	-	++	+	-	-
Polyphenones	++	-	+	-	+	-
Tannins	+	-	+	++	+	-
Terpenoids	+	+	+	+	+	+

*+++ = concentrated, ++ = moderate − = absent * M. E. = Methanolic extraction *A. E = Aqueous extraction*

6.2. Anthelmintic activity of aqueous and methanol extracts of *Allium sativum* (*Garlic*)

6.2.1. Egg Hatch Inhibition Test (EHIT)

Egg hatch inhibition is normally calculated as a function of the number of larvae hatched in proportion to the total number of eggs added. In this study larvated eggs have also been included assuming that they would hatch soon after counting. Accordingly, eggs incubated

with either PBS or DMSO had hatched at >70% while hatchability was reduced to about 15%, 20-24% and 22-35% when eggs were incubated with the positive control (Albendazole 5mg/ml), methanol extract and aqueous extract of *A. sativum* respectively (Figure-5). There was statistically significant difference between control wells and treatment wells ($P<0.000$, $X^2= 56.543$) were as values were statistically similar among the three treatment groups ($P>0.05$, $X^2= 1.928$).

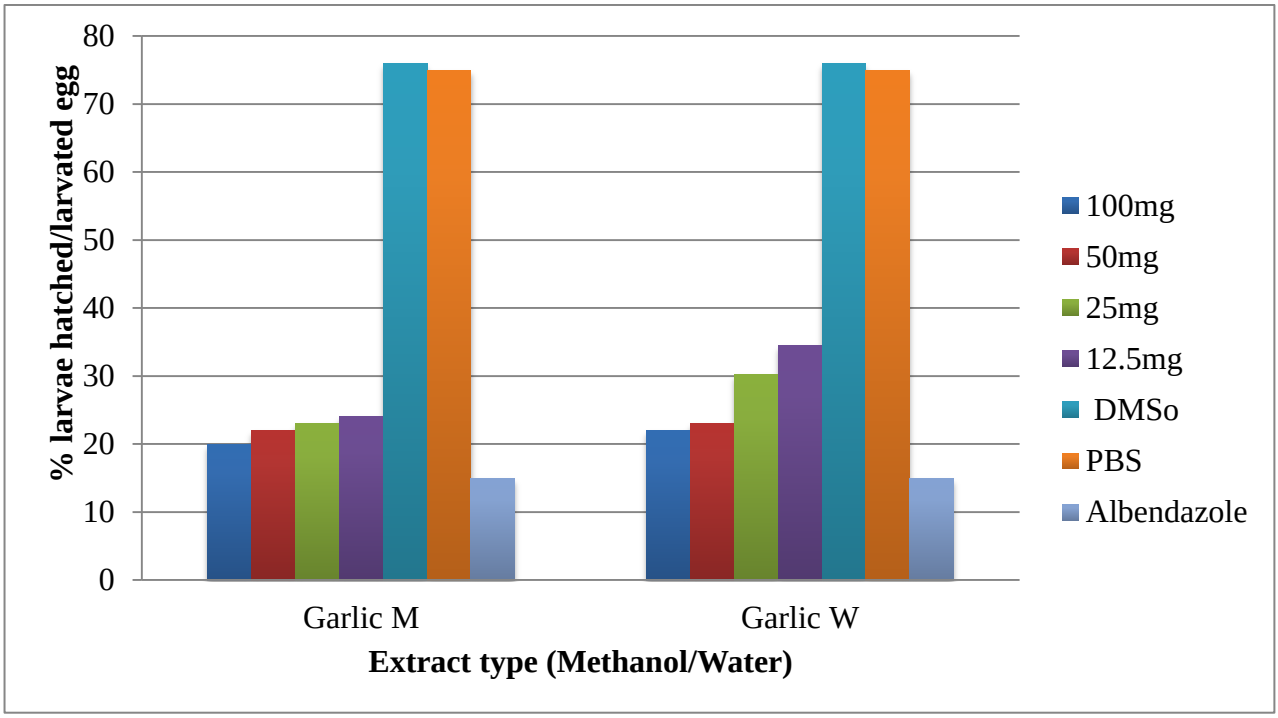

Figure 5-Egg-hatch inhibition effect of different concentrations of Methanol and aqueous extracts of *Allium sativum* on eggs of GIT nematodes

6.2.2. Larval Motility inhibition Test (LMIT)

All concentrations of the methanol extract as well as 200mg/ml and 100mg/ml concentrations of the aqueous extract have larval killing ability which is significantly different from the negative control mixtures ($P<0.0001$, $X^2= 21.227$) whereas no difference was detected between the negative control and the 50mg/ml and 25mg/ml aqueous extract treatment groups after 12 hours of incubation at 27ºC (Figure-6). On the other hand, methanol extract of *garlic* at 200 and 100mg/ml concentrations have identical 100% larval killing ability to the positive control drug, Ivermectin 0.5 mg/ml at the end of 12 hours incubation. Although they have still

significant larvicidal effects, the two higher concentrations of the aqueous extract and the two lower concentrations of the methanol extract had significantly reduced effect compared to the impact of Ivermectin (P<0.0001).

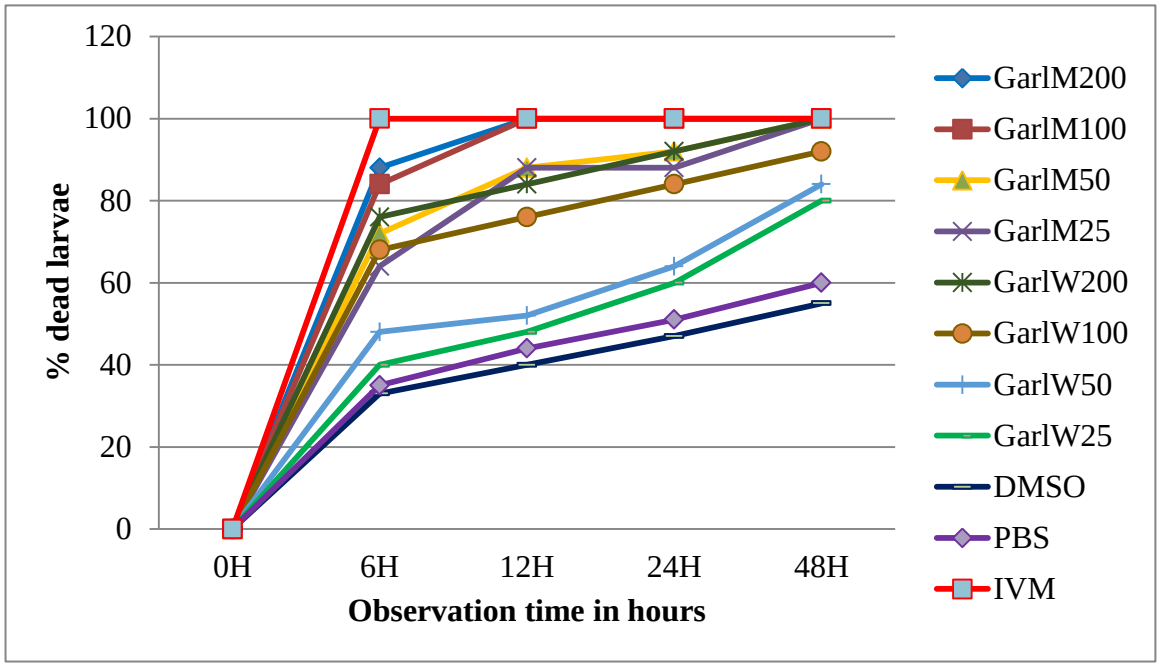

***Figure 6*:** Larvicidal effect of different concentrations of Methanol and aqueous extracts of *Allium sativum* on *Haemonchus contortus* L3 larvae

6.2.3. Adult Motility Inhibition Test (AMIT)

Adult Haemonchus *contortus* freshly collected from abomasa of sheep/goat were exposed to different concentrations of methanol and aqueous extracts of *A. sativum*. The findings show that worm immobility or death was dependent on extract type, extract concentration and exposure time (Figure-7). Accordingly, nine hours after treatment, only 20% of worms with PBS or DMSO were found dead while all the other treatment groups had shown significantly higher death compared to the negative controls (P<0.0001, X^2= 19.681). Nine hours after treatment, methanol extract of *A. sativum* at all concentrations had 100% killing efficacy which is equal to the efficacy of Albendazole while the effect of aqueous extracts remain below 60% throughout the incubation period.

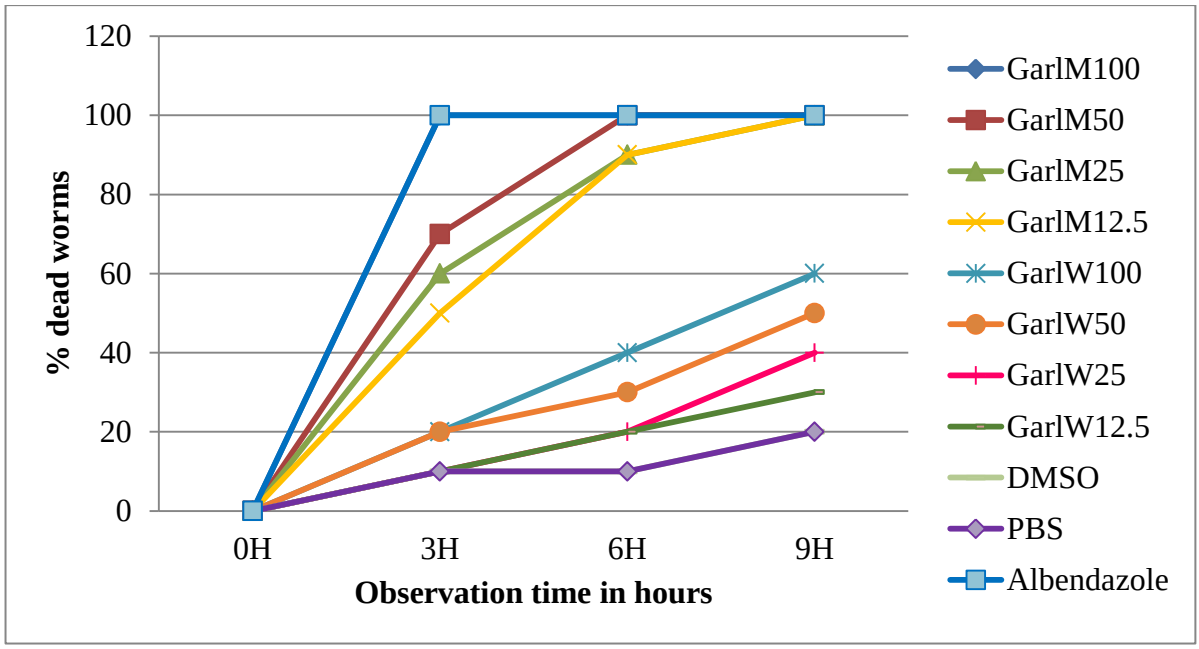

***Figure 7*:** Worm killing ability of different concentrations of methanol and aqueous extracts of *Allium sativum* on adult Haemonchus *contortus*

6.3. Anthelmintic activity of aqueous and methanol extracts of *Zingiber officinale* (*Ginger*)

6.3.1. Egg Hatch Inhibition Test (EHIT)

Gastrointestinal nematode egg hatchability was compared following treatment with different concentrations of methanol and aqueous extracts of *Zingiber officinale* in the presence of positive (Albendazole 5mg/ml) and negative (PBS or DMSO) control groups *in vitro* (Figure-8). The findings revealed that the best (97% inhibition) egg hatch inhibition was with 100mg/ml *Ginger* methanol extract which was statistically higher than the effect of the positive control, Albendazole (P= 0.0031, X^2= 8.747). On the other hand, *Ginger* methanol extract at 50mg/ml and all concentrations of the aqueous extract had comparable egg hatch inhibition effect ranging between (80-85%), (P> 0.05).

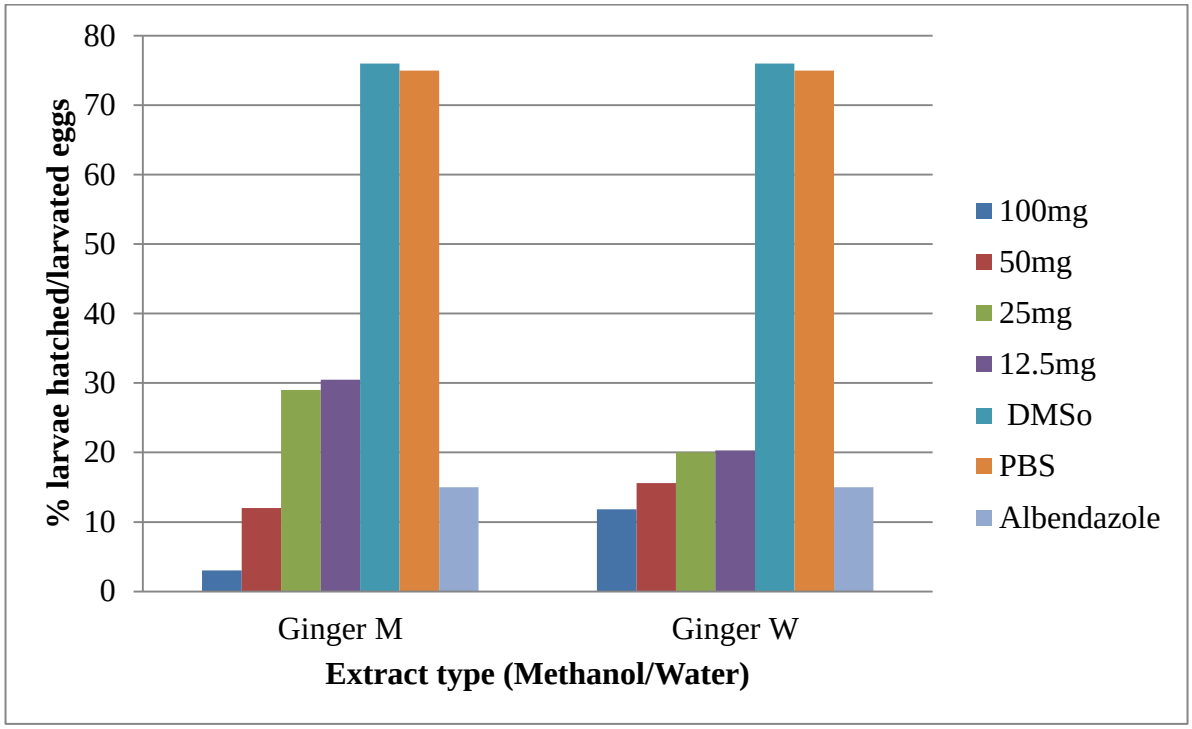

Figure 8: Egg-hatch inhibition effect of different concentrations of methanol and aqueous extracts of *Zingiber officinale* on eggs of GIT nematodes

6.3.2. Larval Motility Inhibition Test (LMIT)

All concentrations of both methanol and aqueous extracts of *Z. officinale (ginger)* had higher larval killing efficacy when compared to the negative control groups ($P < 0.05$, $X^2 = 5.103$). For both types of extracts, larval killing was greater or equal to 92% at 200 and 100mg/ml concentrations with methanol extract at highest concentration having 100% killing efficacy comparable to Ivermectin after 12 hours of exposure. On the 48th hour, all extracts caused greater than 92% larval mortality (Figure-9).

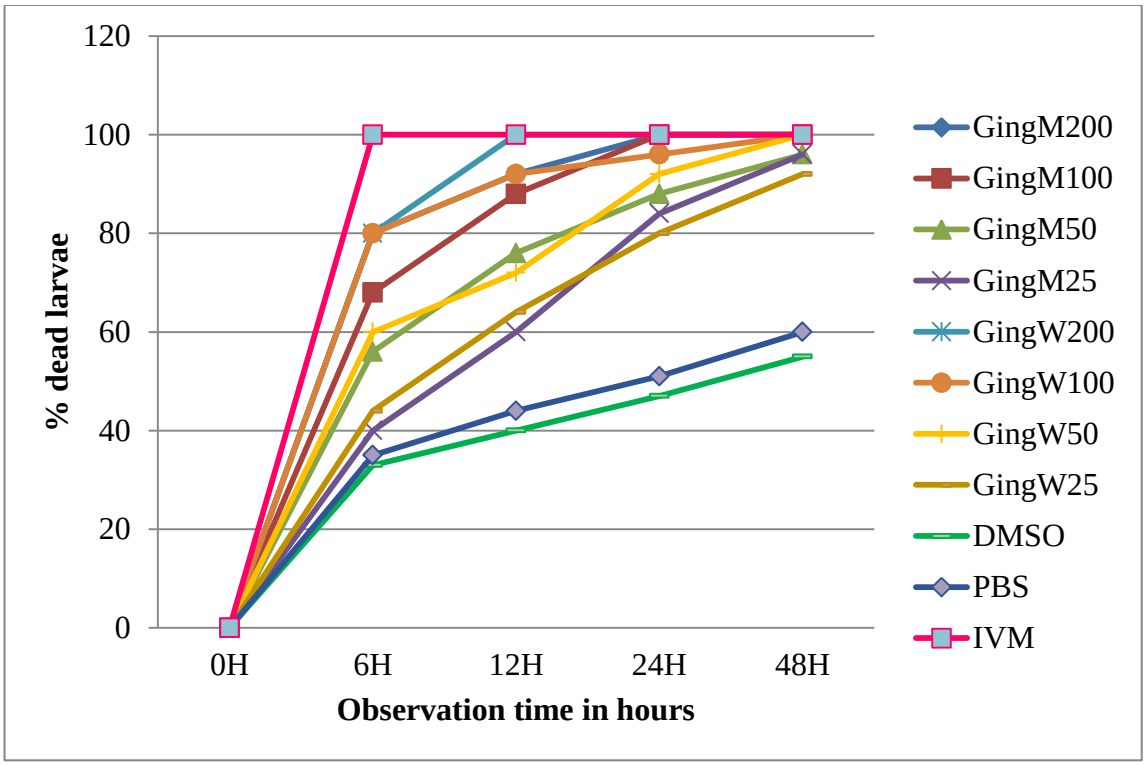

Figure 9: Larvicidal effect of different concentrations of Methanol and aqueous extracts of *Zingiber officinale* on Haemonchus *contortus* L3 larvae

6.3.3. Adult Motility Inhibition Test (AMIT)

Exposure of H. *contortus* adult worms to various concentrations of methanolic and aqueous extracts of *Zingiber officinale* has revealed that except the two lowest concentrations of the aqueous extract, all concentrations including Albendazole treatment had already killed all the worms on the 3rd hour of treatment (Figure-10). Nine hours after treatment, all the treated worms have died while only 20% of them were found dead with the negative control treatments (PBS and DMSO) and the difference between treated and non-treated groups was highly significant ($P< 0.0001$, $X^2= 132.667$).

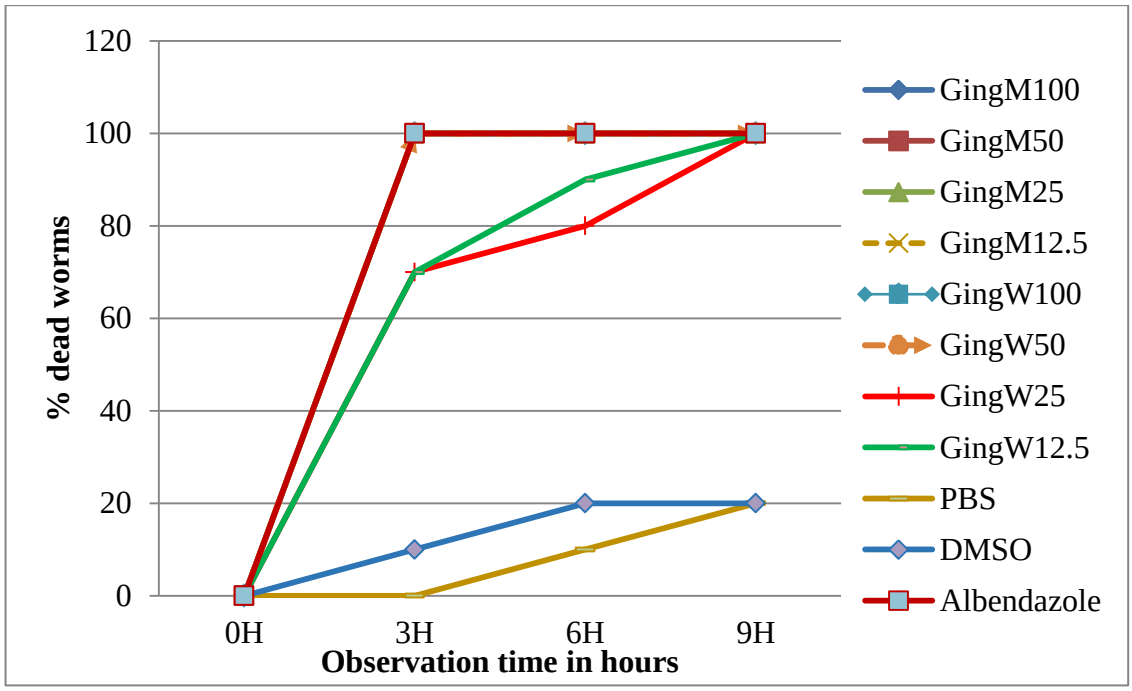

Figure 10: Adult worm killing ability of different concentrations of methanol and aqueous extracts of *Z. officinale* on adult Haemonchus *contortus*

6.4. Anthelmintic activity of aqueous and methanol extracts of *Ruta graveolens* (*Rue*)

6.4.1. Egg Hatch Inhibition Test (EHIT)

Both extract types of *Ruta graveolens* had much lower egg hatch inhibition effect compared to the positive control (Albendazole), as well as extracts of *A. sativum* and *Z. officinale* ($P<0.001$) at most concentrations. While the effect of the methanol extract is detectable compared to the two negative controls, no variation was observed with the aqueous extract (Figure-11).

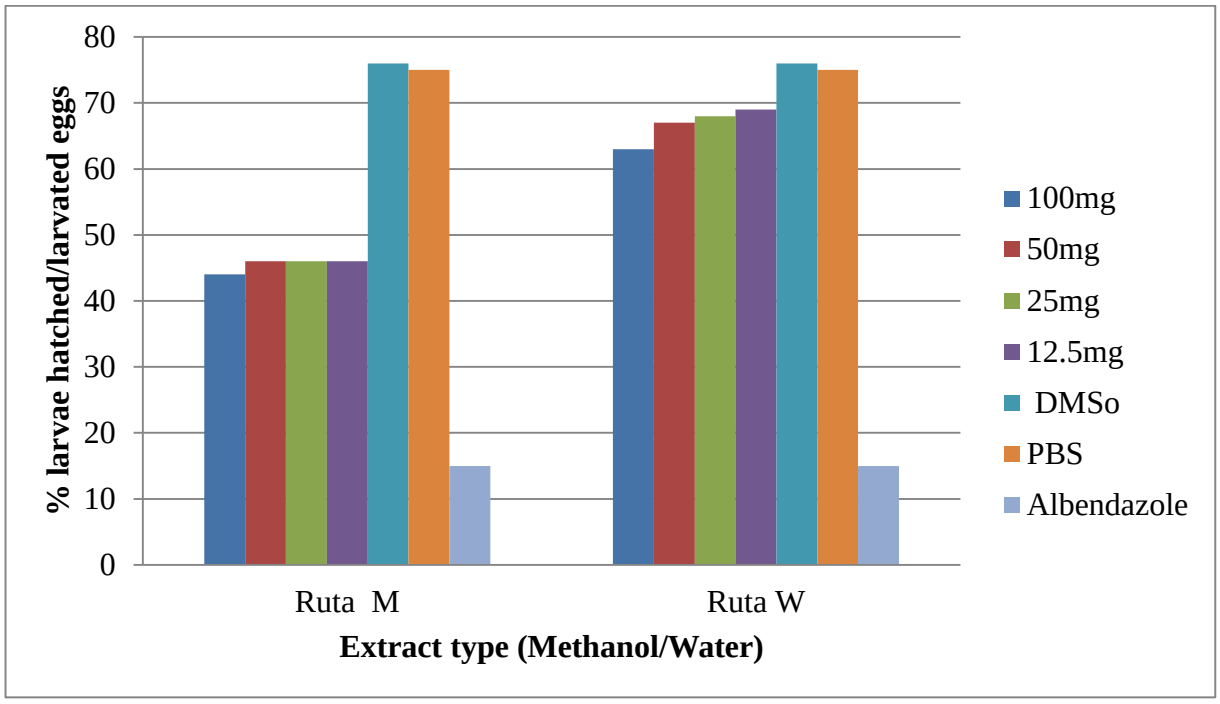

Figure 11: Egg-hatch inhibition effect of different concentrations of methanol and aqueous extracts of *Ruta graveolens* on eggs of GIT nematodes

6.4.2. Larval Motility Inhibition test (LMIT)

The larvicidal efficacy of methanolic and aqueous extracts of leaves of *Ruta garveolens* against *H. contortus* infective larvae was tested at different concentrations *in vitro* and the findings were compared with that of Ivermectin as a positive control and PBS/DMSO as negative controls (Figure-12). Larval counts at 6, 12, 24 and 48 hours show that, larvae in Ivermectin treated wells had no surviving larvae six hours following treatment while larval death increased with increasing time of exposure with all the extract concentrations. It was also observed that, as time passes, significant number of larvae have also been lost even in the absence of treatment. Both methanol and aqueous extracts of the *Rue* leaves have killed 84% of the infective larvae of *H. contortus* at 200 and 100mg/ml concentrations 12 post exposure. This effect was significantly lower than that of Ivermectin ($P< 0.0001$, $X^2= 17.304$) which was 100%.

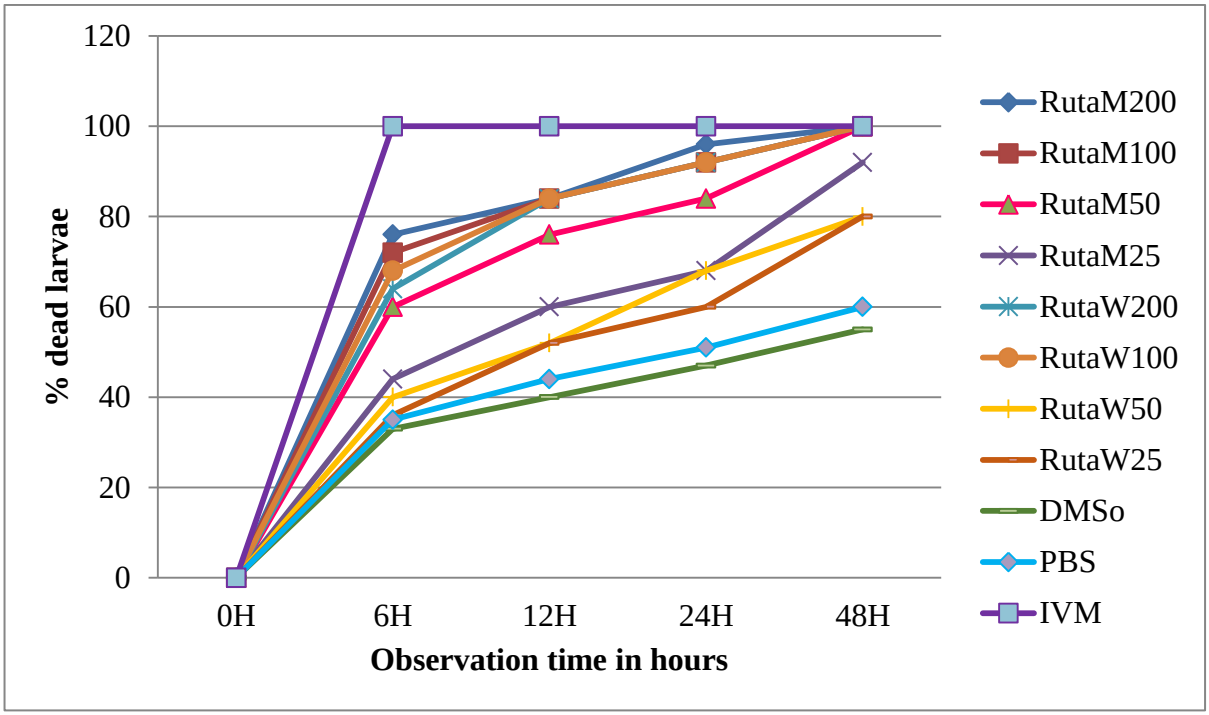

Figure 12: Larvicidal effect of different concentrations of methanol and aqueous extracts of *Ruta garveolens (Rue)* on Haemonchus *contortus* L3 larvae

6.4.3. Adult Motility Inhibition Test (AMIT)

Exposure of *H. contortus* adult worms to various concentrations of methanolic and aqueous extracts of *R. garveolens;* Albendazole and PBS/DMSO being positive and negative controls. No live worm were detected (100% dead) in wells treated with all concentrations of the methanolic extract, Albendazole and wells treated with 100mg/ml of aqueous extract three hours post exposure and the situation remained the same until the end of the experiment. On the other hand, although their effect is still significantly higher ($P<0.0001$, $X2= 98.495$) than the negative controls (50-90% versus 10-20%), efficacy of lower concentrations (50mg/ml and below) of the aqueous extracts never reached 100% at the end of the 9th hour of observation (Figure-13).

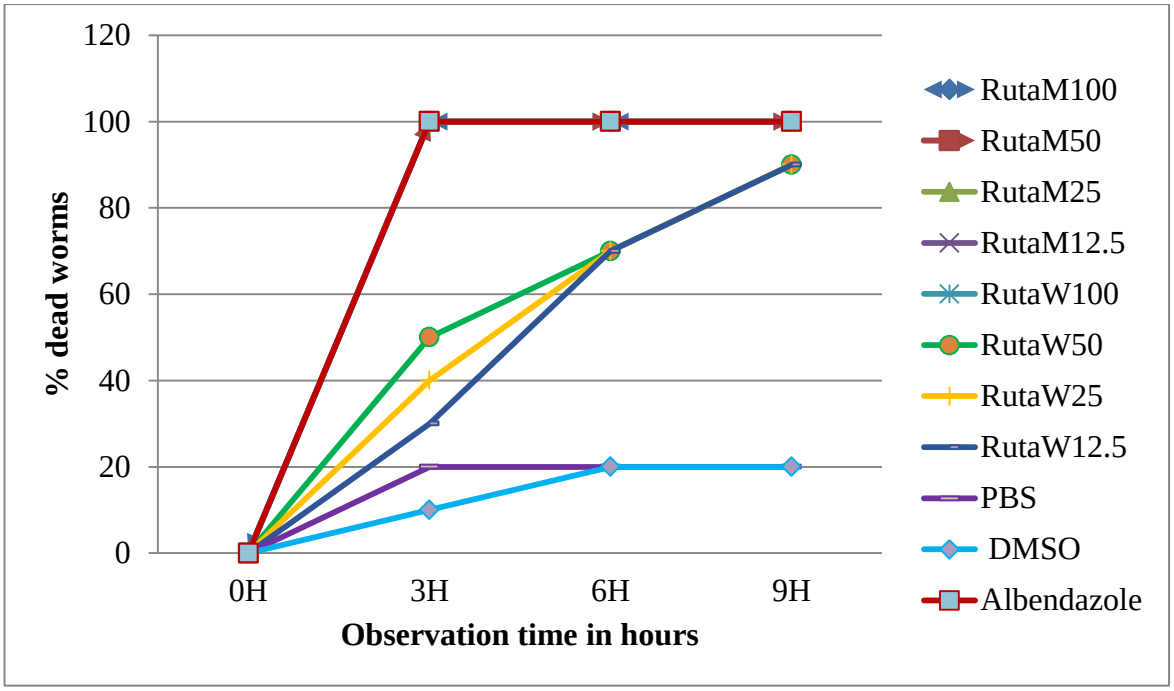

Figure 13: Adult worm killing ability of different concentrations of methanol and aqueous extracts of *R. graveolens* on adult Haemonchus *contortus*

6.5. Comparison of efficacy among the different plants

6.5.1. Against adult H. contortus

Comparative assessment of the effect of methanolic extract of the three plant species shows an outstanding efficacy, equivalent to the commercial drug Albendazole ($P > 0.05$) against adult *H. contortus* worms at all extract concentrations. Especially, Albendazole at 5mg/ml, *ginger* and *Ruta* extracts at concentrations above 50mg/ml have killed 100% of the worms (Figure-14). On the other hand, aqueous extract of *garlic* had killed significantly lower number of worms than the other two extracts as well as the commercial drug ($P<0.05$) at all concentrations (Figure 15).

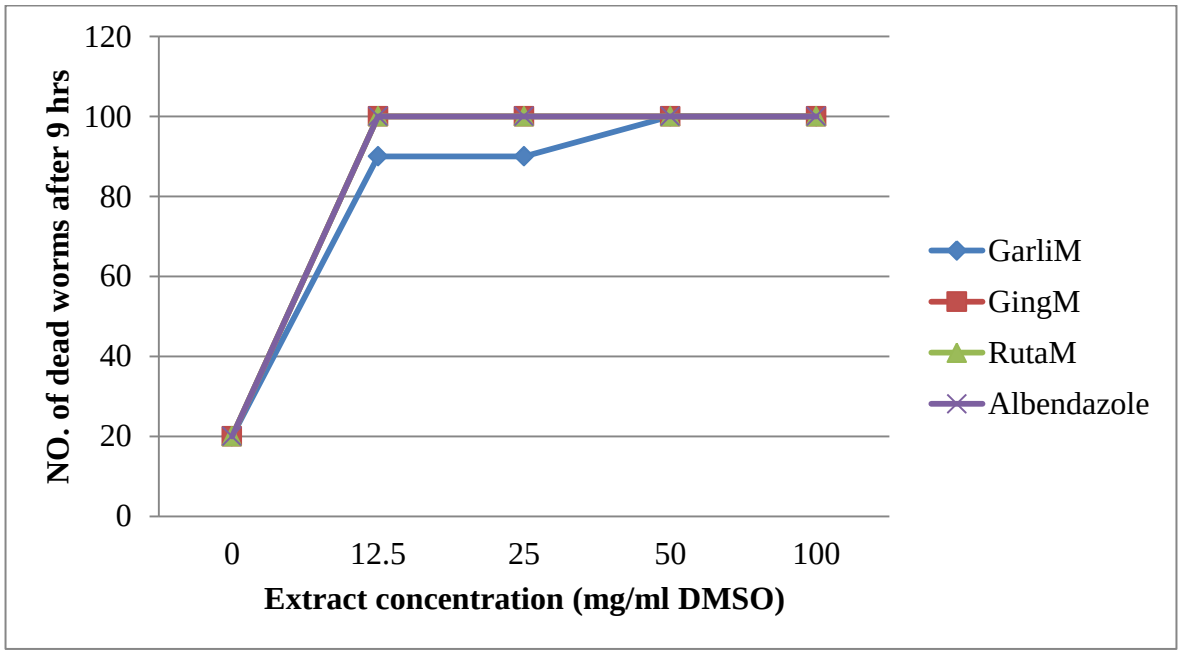

Figure 14: Comparison of the efficacy of methanolic extracts of the three plants against adult *H. contortus* following 9 hours incubation

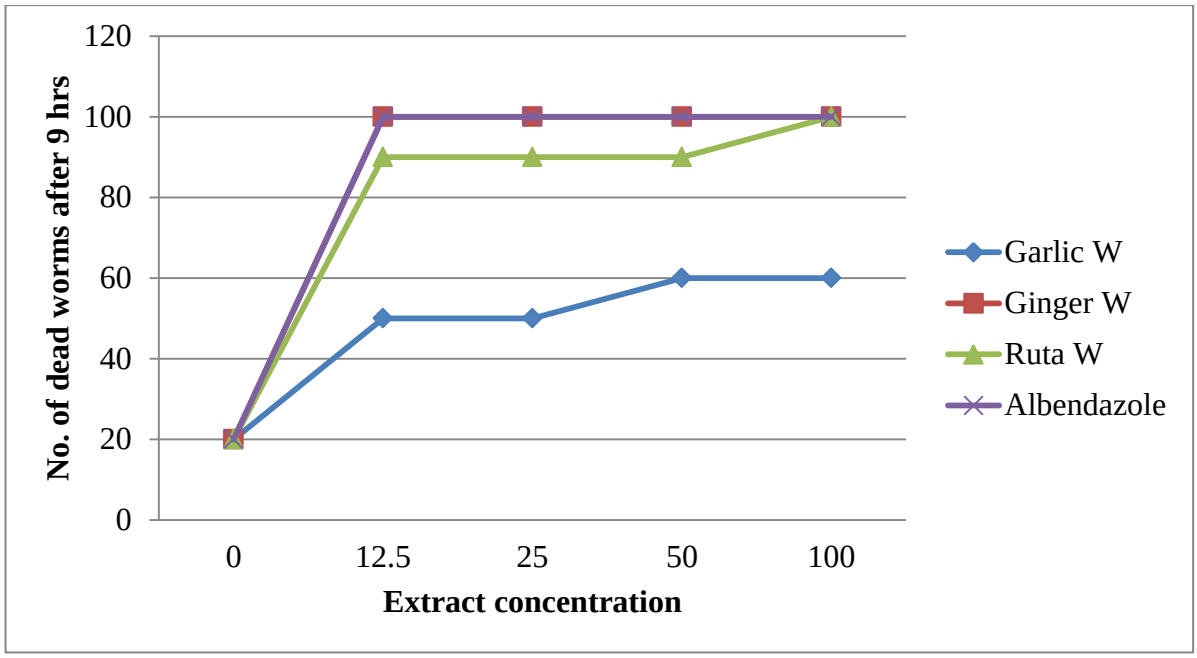

Figure 15: Comparison of the efficacy of aqueous extracts of the three plants against adult *H. contortus* following 9 hours incubation

6.5.2. Against H. contortus L3

Methanol extracts of *ginger* and *Rue* killed less than 80% of the worms at lower concentrations (25 and 50mg/ml) whereas *garlic* had an intermediate effect compared to the commercial drug Ivermectin (Figure-16). In a similar manner, aqueous extracts at lower concentrations of all the three plants had significantly lower effect on H. *contortus* infective larvae compared to Ivermectin which has killed 100% of the larvae (Figure-17). At higher concentrations, almost all extracts had greater than 80% efficacy ($P < 0.005$).

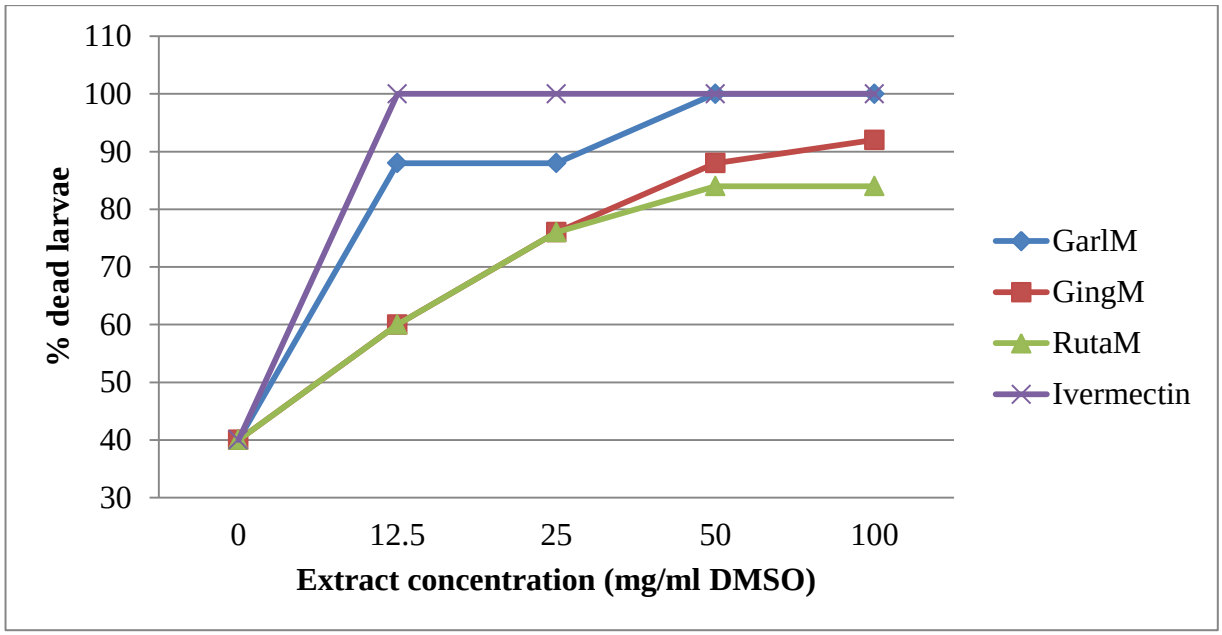

Figure 16: Comparison of the larvicidal effect of methanolic extracts of the three plants against L3 of *H. contortus* following 12 hours of incubation

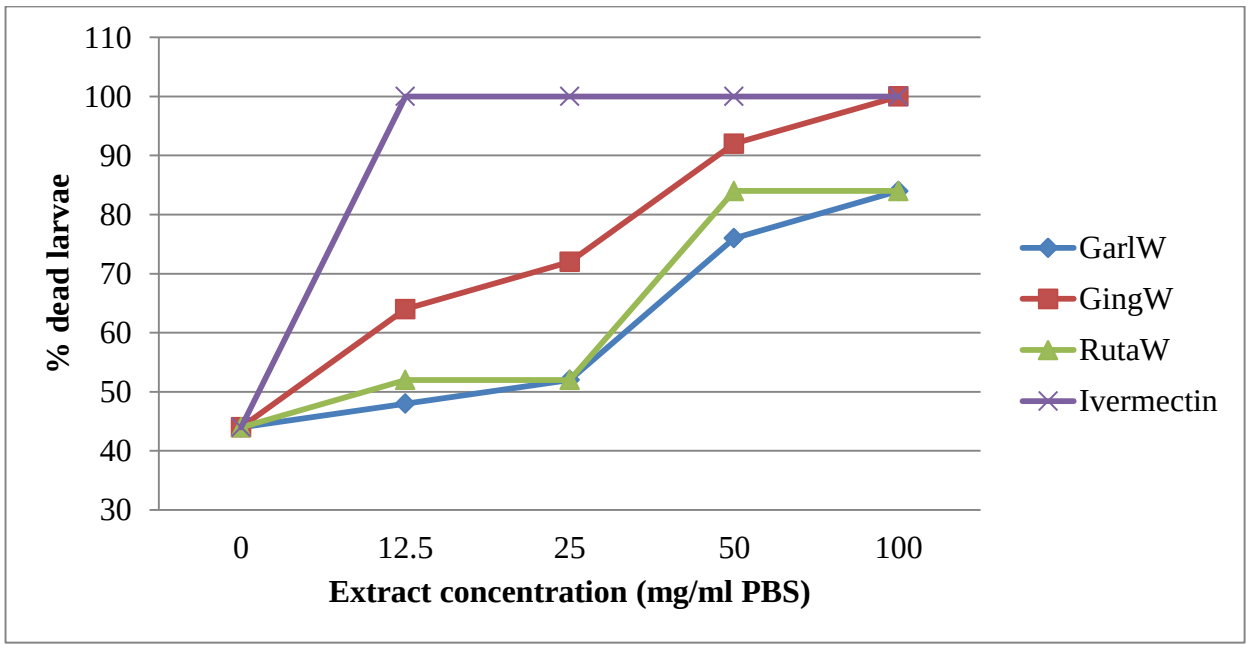

Figure 17: Comparison of the larvicidal effect of aqueous extracts of the three plants against L3 of *H. contortus* following 12 hours of incubation

6.5.3. Against eggs of Gastrointestinal nematodes

Methanolic extract of *Ruta graveolens* had inhibited less than 60% of nematode eggs at all concentrations tested whereas *ginger* extract had shown a concentration dependent increasing efficacy which goes beyond the effect of Albendazole (Figure-18) although the difference was not statistically significant (P >0.05). In a similar manner, aqueous extract of *Rue* had much less ovicidal effect at all concentrations compared to the other two extracts and the positive control while *garlic* had a moderate effect reaching up to 77% with increasing concentration. (Figure-19)

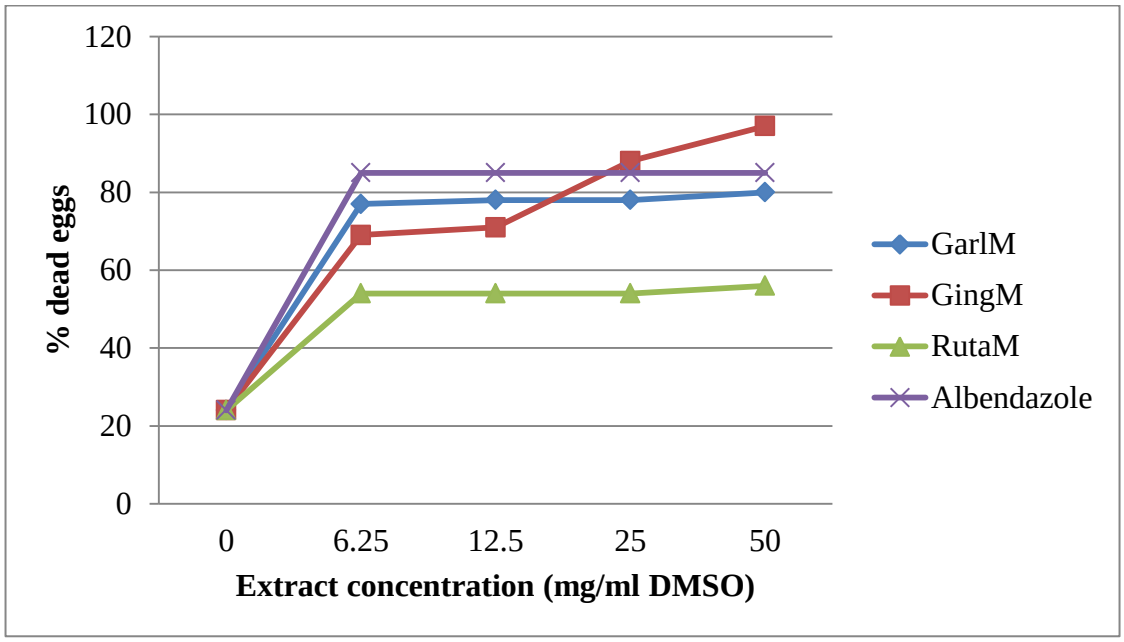

Figure 18: Comparison of the ovicidal effect of methanolic extracts of the three plants against eggs of GIN following 48 hours of incubation

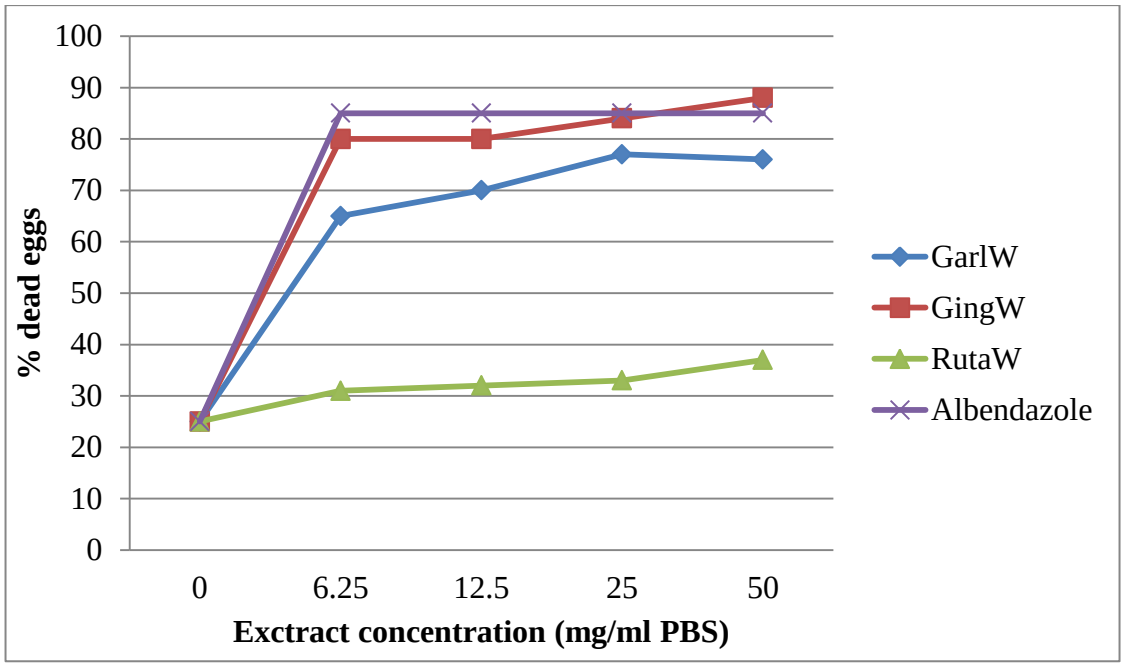

Figure 19: Comparison of the ovicidal effect of aqueous extracts of the three plants against eggs of GIN following 48 hours of incubation

6.6. Association of extracts' phytochemical contents and efficacy

As is already presented in table 6 above, extracts of the three plants varied in their contents of secondary metabolite. It was observed that even when similar compounds are present the activity varied between plants and extract types. In general, extracts that have shown strong activity against adult *H. contortus* were found to have higher concentrations of glycosides, steroids, flavonoids, polyphenols, tannins or saponins. On the other hand, extracts with higher activity on the infective stage (L3) of the parasite had abundance of glycosides and saponins. Saponins, polyphenols and alkaloids are associated with moderate activity in reducing egg hatchability while glycosides show strong association in this respect.

Table 7: Relative abundance of secondary metabolites in methanolic and aqueous extracts of *garlic*, *ginger* and *rue*

Plant	Extraction medium	Phytochemical detected in high amount		Efficacy
Garlic	Methanol	Glycosides		Strong on all
	Aqueous	Saponins	Alkaloids	Moderate on only eggs
Ginger	Methanol	Saponins	Polyphenols	Strong on adult, moderate on eggs
	Aqueous	Saponins		Strong on adult and eggs, moderate on L3
Rue	Methanol	Steroids		Strong on adult
	Aqueous	Flavonoids	Tannins	Strong on adult

7. DISCUSSION

Scientific validations of anthelmintic activities of different natural and synthetic compounds have often been based on *in vitro* and *in vivo* studies. *In vitro* studies have been conducted to see the effects of plant extracts on parasite egg hatchability, motility and survival of larval and adult worms of animals and man including *Haemonchus contortus* of small ruminants, *Ascaridia galli* of poultry and *Ascaris lumbricoides* of man (Lateef *et al.,* 2003). The assumption made in these *in vitro* studies is that the intensity and types of activity observed against the model nematodes will be similar in infected livestock and man. Hence such tests are valuable for initial screening for further animal testing (Pessoa *et al.,* 2002).

This *in vitro* study has evaluated the effect of methanolic and aqueous extracts of *Allium sativum, Zingiber officinale* and *Ruta graveolens* on nematode egg hatchability and motility/survival of infective stage and adult *H. contortus* as a model parasite species. As different species of plants may contain different types and concentrations of active secondary metabolites, it is expected that extracts from different plants could vary in their efficacy against pathogens including helminth parasites. In this study it was observed that except for aqueous extract of *garlic*, all extracts had excellent efficacy against adult H. *contortus* at all times tested. Iqbal *et al*. (2001) compared the efficacy of *Allium sativum, Zingiber officinale, Curcurbita mexicana* and *Ficus religiosa* against *H. contortus* adult worms. They confirmed that extracts from *ginger* killed 100% worms at 2 hours post exposure while a similar effect with *garlic* occurred after six hours implying that although exposure time has some effect on the activity of the extracts, both are excellent candidates as anthelmintics.

7.1. Efficacy of *Allium sativum* (*Garlic*) on eggs, L3 and adult *H. contortus*

Allium sativum, commonly known as *garlic* has been cited for its use as a medicinal plant against various ailments of man and animals (Imo and Za'aku, 2019). In this study, methanolic extract of *garlic* has shown strong efficacy against the egg, larval and adult stages of the nematode, *H. contortus* in a concentration and time dependent manner while the aqueous extract had only moderate effect on egg hatchability. The effect methanolic extract on adult parasites, three hours after exposure at 100mg/ml and nine hours post exposure at all

concentrations tested, was equal to that of the commercial drug (Albendazole) strongly suggesting that the extract has potent active metabolites toxic to the parasites. This is in line with an *in vivo* study by Kumar *et al.*, (2017) who have also observed that orally administered ethanolic extract of *garlic* bulb revealed 85% efficiency in naturally *Haemonchus contortus* infected sheep whereas aqueous extract revealed moderate efficiency (56%) on the basis of Egg per Gram (EPG) reduction. An earlier *in vitro* study by Iqbal *et al., (*2001) also substantiated that the extract of *allium sativum* was found 100% effective against adult *H. contortus* in less than six hours of exposure. The difference in efficacy based on extraction solvent and the stage of the parasite, through requires further investigation, may imply that different secondary metabolites or different concentrations of the compounds are recovered by the two extraction methods. Water being more polar (polarity=1) than methanol which has a polarity of 0.762 (Christian and Thomas, 2010), the two solvents may vary in their ability to pick different active compounds out of the plant material.

Contrary to the current observation, the 100% *in vitro* efficacy three hours after exposure reported for aqueous extract of the plant at 100mg/ml against T*richostrongylus*, *Ostertagia* and *Haemonchus* species collected from cattle in Bangladesh (Amin *et al.*, 2009) may suggest difference in either the sensitivity of the parasites to compounds available in the extracts or variation in concentration or type of active metabolites available as the sources of the plants are from to geographically distant origins.

Magdeleine *et al*. (2010) reported that methanolic extracts of plants contain a large spectrum of compounds that have a multiple target activity on eggs to reduce their hatchability. However, despite the difference in abundant active metabolites detected between the two extracts in this study, no significant difference was observed on the inhibitory effect of both types of extracts 48 hours post exposure at all concentrations tested. This requires further investigation as the details of chemical compounds present.

Kumarasingha *et al*., (2016) reported that whole plant and root extract of *Picria fel-terrae Lour* had significant larval immobilization effect on *H. contortus* L3. In a similar manner, Palacio-Landín *et al.,* (2015) documented that the lethal concentration (LC50) for *Allium sativum* extract against L3 of *H. contortus* was 3.8 mg/ml. in support of these observations; the current study revealed 100% mortality of *H. contortus* L3 at all concentrations of the

methanolic extract and greater than 80% mortality with 25-100mg/ml of the aqueous extract 48 hours post exposure. This suggests that compounds in *garlic* had potent activity against the larval stage of the parasite. However, since L3 in the host is excheathed in the rumen and passes to the abomasal mucosa within hours, if this product has to be effective against the larvae *in vivo*, it has to act earlier than 48 hours. In this regards, at 12 hours of exposure, the 50 and 100mg/ml of methanol extract produces 100% larval death while only the 100mg/ml aqueous extract of *garlic* results in over 80% death suggesting that the action is both concentration and time dependent.

The overall observation is that the mode of absorption and action of the chemical compounds vary based on the stages of the parasites. Sisay *et al.,* (2012) reported that extract types of plants that are effective against one developmental stage of the parasite may not be effective against other developmental stage.

7.2. Efficacy of *Zingiber officinale* (*Ginger*) on eggs, L3 and adult *H. contortus*

One of the most commonly used plants which have been tested for its anthelmintic properties is *ginger* (*Zingiber officinale*). Rhizomes of this plant have been demonstrated to have efficacy against larval stages of *Angiostrongylus cantonensis* and *protoscolices* of hydatid cyst *in vitro* as well as against gastrointestinal nematodes of sheep and pig *in vivo* (Lin *et al.,* 2010; Moazeni and Nazer, 2011; Kiambom *et al.,* 2020; Iqbal *et al.,* 2006). It has also been shown that different varieties may contain different concentrations of active metabolites which may vary depending on geographic locations from which the plants are collected (Juliani *et al.,* 2007).

This study has unequivocally shown that both methanolic and aqueous extracts of *ginger* have moderate to high efficacy against eggs of nematodes and adult *H. contortus.* This means that, the extracts have the potential to be active *in vivo*; on adults in the abomasum and eggs in the intestines. In line with this finding, Iqbal *et al*. (2001) reported 100% mortality of adult *H. contortus* within 2 hours of exposure to the extract of *Z. officinale*. In the current study, all concentrations of the methanol extract and the highest concentration of the aqueous extract have already killed 100% of the worms three hours post exposure suggesting that the action is very rapid. The findings of Iqbal *et al.,* (2006) also show that both crude powder and aqueous

extract of the plant exhibited a dose and time dependent anthelmintic effect on EPG when drenched to sheep. In addition, its powder was reported to reduce 92.6% FEC against *Strongyloides ransomi* of pigs (Kiambom *et al.,* 2021). Ethyl acetate extract of *ginger* has also been reported to kill adult *Shistosoma mansoni in vitro* (Sanderson *et al.,* 2002). Similarly, Moazeni *et al*., (2016) have shown experimentally that extracts of *Zingiber officinale* have potent ovicidal effect against *Fasciola hepatica* eggs *in vitro*.

Chemical analysis of *ginger* shows that it contains over 400 different compounds where the main components of *ginger* rhizomes are carbohydrates (50–70%), lipids (3–8%), terpenes, and phenolic compounds. *Ginger* extracts used in this study had saponins and polyphenols in large amount. Products of this plant have also been reported to have antibacterial effect (Teles *et al.,* 2019) which needs further study to know if oral dosing by this extract undermines the function of ruminal micro flora.

7.3. Efficacy of *Ruta graveolens (Rue*) on eggs, L3 and adult *H. contortus*

Ruta graveolens (*rue*) is commonly used plant that is presented for several biological activates such as antioxidant, antifungal, phytotoxic, antidepressant and anti-inflammatory (Asgarpanah and Khoshkam, 2012). Some reports on its efficacy have also shown potent anthelmintic activity. Pandey *et al*., (2010) have tested alcoholic extracts of *Ruta graveolens* L. leaves against Indian earthworms (*Pheretima posthuma*) where significant paralysis was observed. A closely related species, *Ruta chalepensis* extract had also shown anthelmintic efficacy *in vitro* (Ortu *et al.,* 2016).

The present study was able to demonstrate that both extract types of *rue* have strong efficacy against adult stage of the abomasal parasite, *H. contortus* implying again that it has also the potential to act *in vivo* provided it resists degradation by digestive enzymes and ruminal fermentation process; which in fact needs further study. Similar to the case for *ginger*, all concentrations of the methanol extract and the highest concentration of the aqueous extract of *rue* killed 100% of the worms three hours post exposure. On the other hand, little larvicidal and ovicidal effect was detected at all concentrations compared to their action against adult parasites supporting the assumption that the mechanism of uptake of plant compounds into the different stages of helminth parasites varies. *Rue*, is known to be a rich source of secondary

metabolites such as coumarins, alkaloids, volatile oils, flavonoids, and phenolic acids (Asgarpanah and Khoshkam, 2012). Phytochemical screening in this study also revealed the presence of steroids, flavonoids and tannins as major constituents although others are also available.

7.4. Secondary metabolites and their possible roles

A primary metabolite is a kind of metabolite that is directly involved in normal growth, development, and reproduction of a plant. It includes vitamins, carbohydrates, proteins, lipids, hormones and nucleic acids. On the other hand, secondary metabolites are not essential as primary metabolites as these are not directly involved in the indicated functions. They are commonly derived from primary metabolites (Pott *et al*., 2019).

Phytochemical analysis of methanolic and aqueous extracts of different plants show presence of different types of secondary metabolites in different concentrations which are considered as the source of chemical components responsible for wide therapeutic activities of several medicinal plants (Debella, 2002). Different extraction protocols are followed in herbal medicine preparation, ethanol – water mixture extraction protocols are used in majority of herbal medicine industries (Ganora, 2008). In the present study, variations of secondary metabolites were observed between different plants and within the plant with different types of extraction. The observed variation in the constituents of aqueous and methanol extract of the plants could be due to difference in chemical solubility of the components in different solvents. In addition, environmental/geographic factors may also determine availability and concentrations of phytochemical contents in the same species of plant (Kokate *et al.,* 2004).

Qualitative judgment of the chemical composition of extracts in this study shows that *garlic* contains abundant saponins, alkaloids and glycosides. This finding is supported by the study conducted by Mikail (2010) who reported that aqueous extracts of *garlic* was found positive for flavonoid, saponnin and alkaloid whereas Ali and Ibrahim (2019), described the presence of tannin and steroids in ethanol extract of *garlic* bulbs. *Ginger* has more saponins, flavonoids and polyphenols which is in agreement with Abdullahi *et al*., (2017) who have also reported that *ginger* extract were positive for flavonoids, saponins, terpenoids, and polyphenols. Similarly, Adesola *et al*., 2021) have also reported that methanol extract of *Z. officinale*

contain Alkaloids, Steroids, Flavonoids and Tannins. However, steroids were not found in the present finding, this might be due to difference in the geographical area where the plant grew, the differences in the solvents used for extraction or parts of the plant extracted (Solomon *et al*., 2013; Kokate *et al.,* 2004).

Similarly, *Ruta graveolens* was found to be rich in flavonoids, steroids and tannins. Other studies have demonstrated that *Ruta graveolens* methanolic extracts are rich in alkaloids, terpenoids and saponins (kuzovkina *et al*., 2004) and (Hashemi *et al*., 2011). Similarly, other study showed that aqueous extract of leaves of *Ruta graveolens* contained phenolic compounds, alkaloids, steroids, and terpenoides (Kuzovkina *et al*., 2004, Ivanova *et al*., 2004). Also, Hashemi *et al.* (2011) have reported that methanol and chloroform extract of *Ruta gravenoles* contains Saponin and Flavonoids such as rutin and quercetin.

Some of these secondary metabolites may account for the anthelmintic properties of some medicinal plants. Alkaloids may affect DNA replication and cause neurotoxicity followed by paralysis on helminth parasites (Wink, 2012; Roy *et al*., 2010). Molan *et al*., (2003) described that terpenoids have the ability to stop the adult motility and the consequent migration ability of ovine nematode parasites, by binding to surface molecules (proteins or sterols) inducing inhibition of the protein expression, and lyses of the cell. Steroids have also been demonstrated to kill the blood feeding parasite, *Schistosoma mansoni in vitro* (Krieg *et al*., 2017). Similarly, Saponins from the forage crop Medicago species caused 100% inhibition of gastrointestinal strongyle egg hatchability and the effect was comparable to the commercial drug thiabendazole (Maestrini *et al*., 2020). Altogether, the above facts strongly suggest that if the active metabolites are purified and further characterized, they may be potential sources of novel anthelmintics in the future.

8. CONCLUSION AND RECOMMENDATIONS

The present study was initiated to investigate the anthelmintic activates of methanolic and aqueous extracts of *Allium sativum, Zingiber officinale* and *Ruta graveolens* on nematode egg hatchability and inhibition of motility/survival of infective stage and adult *H. contortus* as a model parasite species. The finding revealed that the Aqueous and Methanol extracts of the plants used in the current study showed dose and exposure time-dependent anthelmintic activity. Moreover, the Methanol extract possessed better anthelmintic efficacy. Both types of extracts from *ginger* showed excellent efficacy against nematode eggs while *Ruta* graveolens had the lowest effect among the three plants. All extracts of the three plants, excepting the aqueous extract of *garlic*, had outstanding killing effect against adult *H. contortus*. These suggest that, these extracts can further be tested *in vivo*. *H. contortus* larvae were also highly susceptible to aqueous extracts of *ginger* and moderately to all methanolic extracts of the three plants. Both extracts of the plants were screened for preliminary (qualitative) phytochemical analysis and found to possess various types of secondary metabolites, especially saponins, phenols, glycosides and alkaloids which are reported to be responsible for the anti-parasitic activity.

Therefore, the following recommendations are forwarded for further study and future exploitations of this potential.

- The compounds in the plants should be fractionated and tested both *in vivo* and *in vitro* to evaluate their roles individually and in combination
- *In vivo* evaluation of the plant extracts should be conducted in different animal models in order to assess the toxicity on animals and effect on the function on ruminal micro flora.
- In this study, adult parasites exposed to all the extracts tested were already dead three hours post exposure. Therefore, the efficacy of these extracts before three hours should further be evaluated.

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ANNEXES

Annex 1: Methods used for phytochemical screening of plant extracts

1. **Test for alkaloids:** This was carried out as described by Rafauf (1970)
   a. Dragendorff's test: 1 ml of Hydrochloric acid (HCl) and 3 drops of Dragendorff's reagent were added to the extract solution. The formation of orange precipitates indicated the presence of alkaloids.
2. **Test for anthraquinones**:
   a. Free Anthraquinones: 5 gm of each plant extract was shaken with 10 ml of benzene and filtered. A 10% ammonium hydroxide solution (5 ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonia phase was taken as an indication of the presence of anthraquinones.
3. **Test for saponins**

   a**. Foam test**: To 1 ml of each extract, 3 ml of water was added and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result (Evans, 1996).
4. **Test for sterols**

   Liebermann-Burchard's Test: To 1ml of the extract, few drops of conc. H_2SO_4 acid in a slanty position was added and left standing for an hour. The formation of brown ring at interphase indicates the presence of sterols (Briggs, 1970).
5. **Test for tannins**

   Two milliliters (2ml) of crude extract was mixed with 2ml of 2% solution of ferric chloride ($FeCl_3$). A blue-green or black coloration indicated the presence of tannins.
6. **Test for flavonoids:** This was carried out as described by Dermarderosian and Liberti. (1988)

   Ferric chloride test: Few drops of ferric chloride were added to the extract test solution. Formation of blackish red color indicated the presence of flavonoids.
7. **Test for glycosides**: This was carried out as described by (Evans, 1996): Two ml of crude extract was mixed with 2 ml of chloroform. Thereafter, 2 ml of concentrated sulfuric acid (H2SO4) was added carefully and shaken gently. A reddish brown ring at interphase indicated the presence of glycosides.

8. Test for polyphones (Ferric Chloride test)

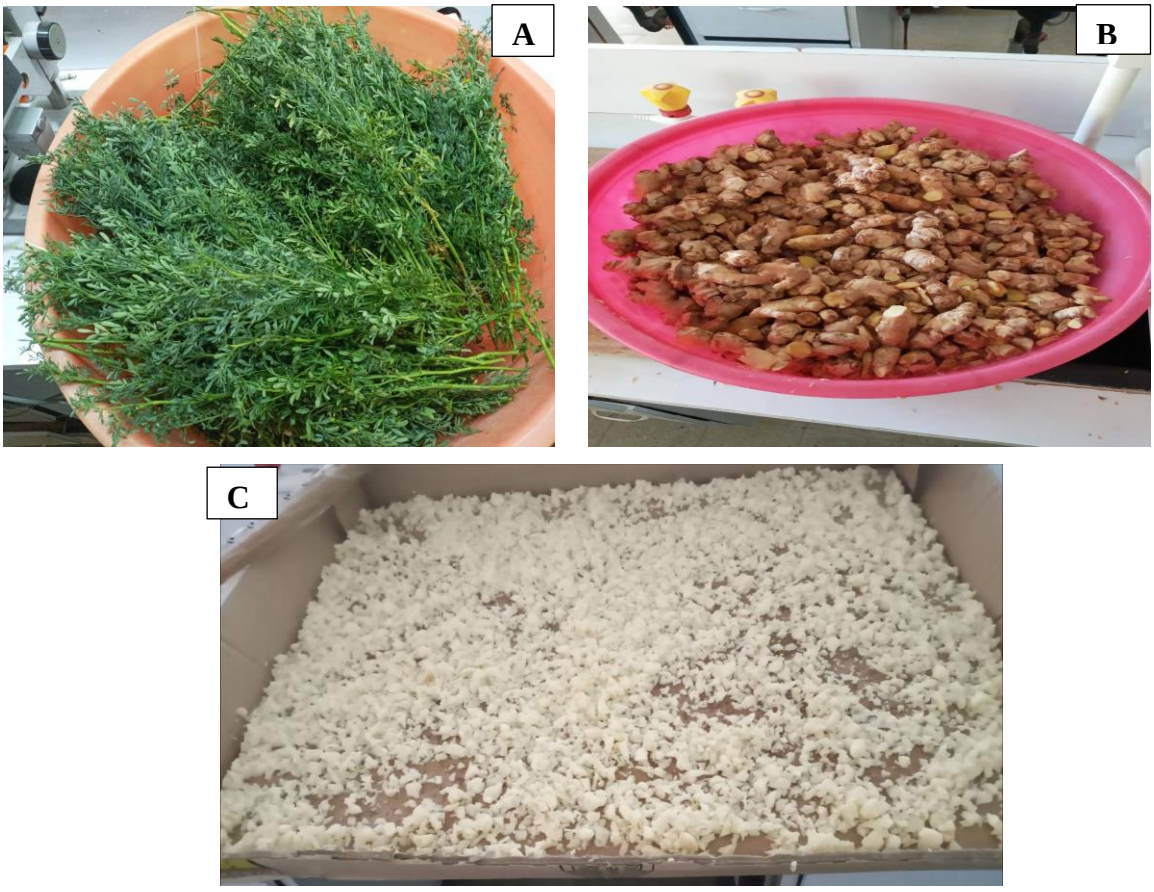
About 5 ml of the crude extract was taken and 1 ml of FeCl3 (1%) and 1 ml K3 (Fe (CN) 6) (1%) were added. The appearance of fresh reddish blue color indicated the presence of polyphenols

9. Test for terpenoids (Salkowski test)

About 0.25 g of each of the plant extract was taken and 2 ml of chloroform was added. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids

Annex 2: Pant material preparation

- Collected plant parts were washed with distilled water to remove dirt and soil particles and carefully inspected for gross lesions or damage.



Fresh leaves of *Ruta graveolens* (A), rhizomes of *Zingiber officinale* (B) cloves of *Allium sativum* (C)

- The plants were cut into small pieces, spread out on paper sheets, dried in shaded area at room temperature for ten days.
- Completely desiccated plant part powdered finely.

Air drying of the study plant material under shade in laboratory

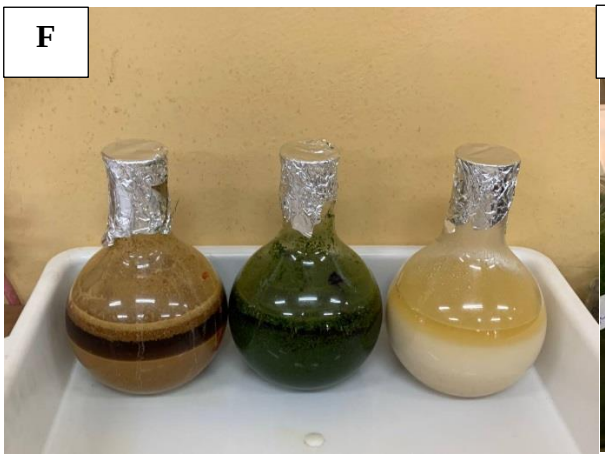
Weighting of powdered plant material for maceration

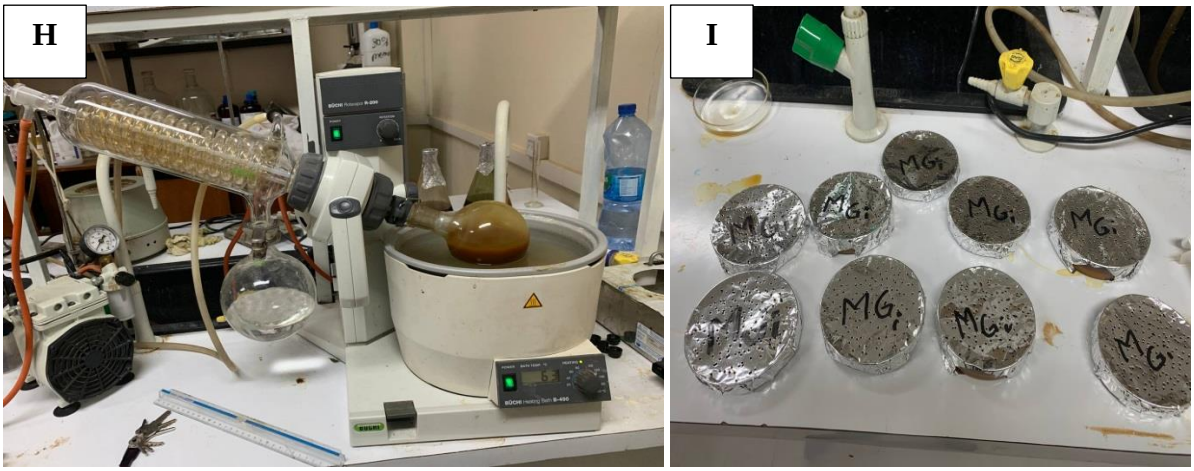
Annex 3: Extraction procedure

- Powdered plant material Weighed, transferred in separate flask and extracting agent added (Methanol and Distil water).
- Each flask containing plant material and extracting agent shaken for 72h intermittently.
- The mixture was later strained using a muslin cloth and filtered using a Whatman filter paper (No. 1: 125mm).
- The filtrate was concentrated in a vacuum rotary evaporator and was evaporated to dryness in an air oven at 40°C

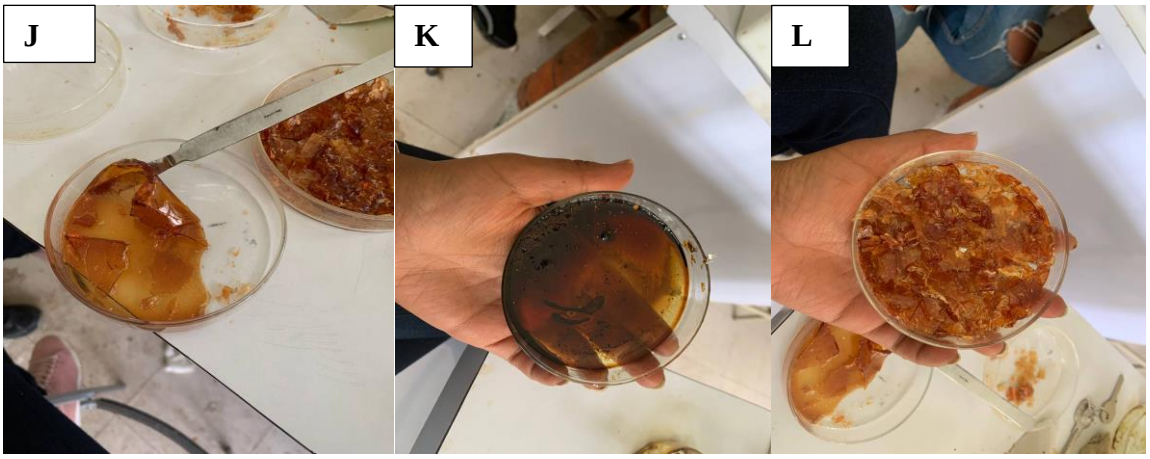
Picture showing straining of mixture of the plants using a muslin cloth (D) and filtered using a Whatman No.1 filter paper (E)

Annex 4: Maceration technique of plant material with extracting agents (Metahnol (F) and Distilled water (G)) used for maceration

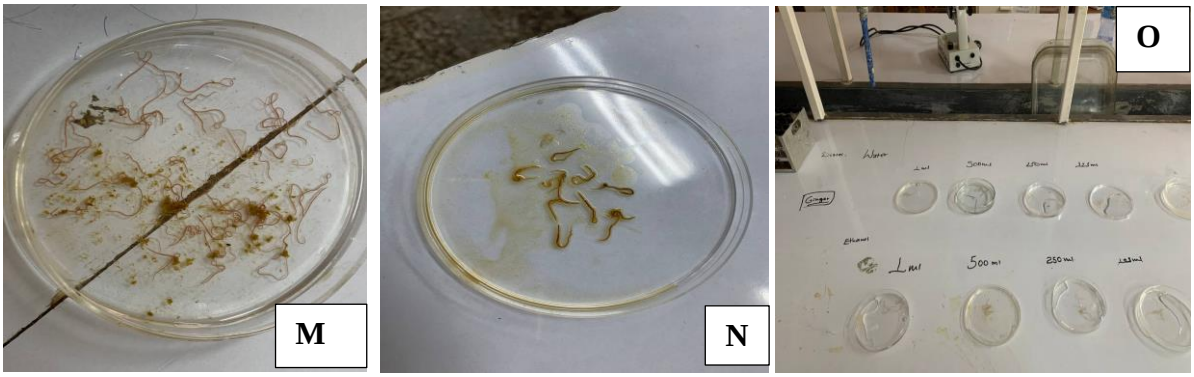




Picture of Rota Vapor (extraction equipment) (H) and Prepared extract for further drying in the oven (I)



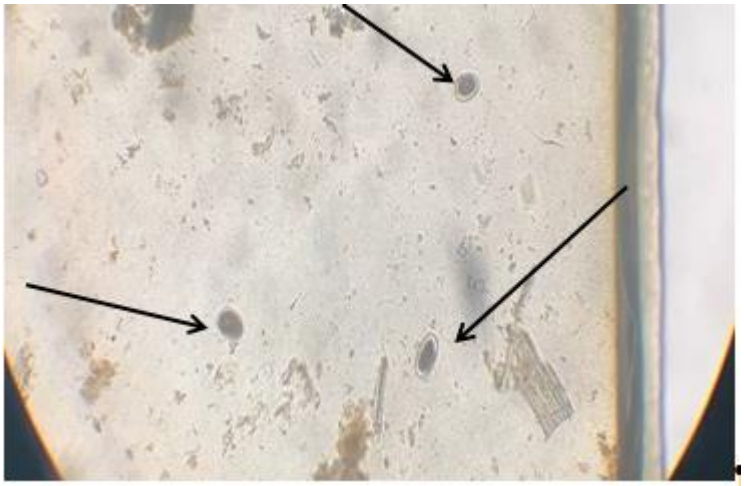
Extract scraping off after drying in the oven *Ginger* (J), Tena adam (K), *Garlic* (L)



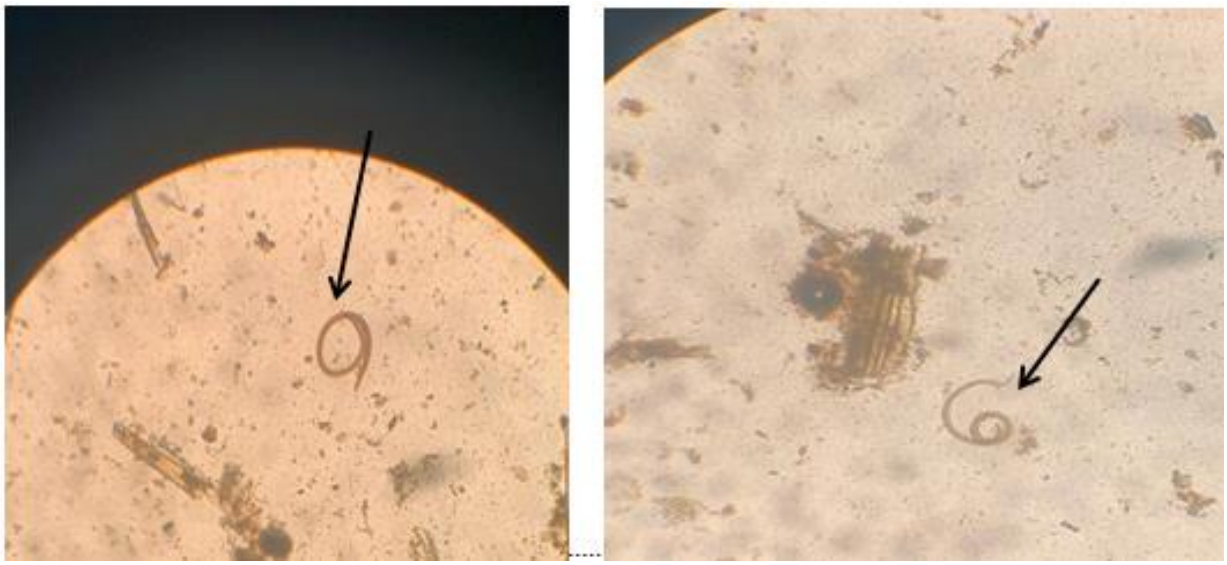
Annex 5: Picture showing Live adult Haemonchus contortus suspended in PBS after collection (M) and exposed in different concentration of plant extract (N, O)

Annex 6: Picture showing fecal culture at room temperature (left) and recovery of infective larva (right)

Incubated eggs (H) and larvae (I) with different concentration of plant extracts

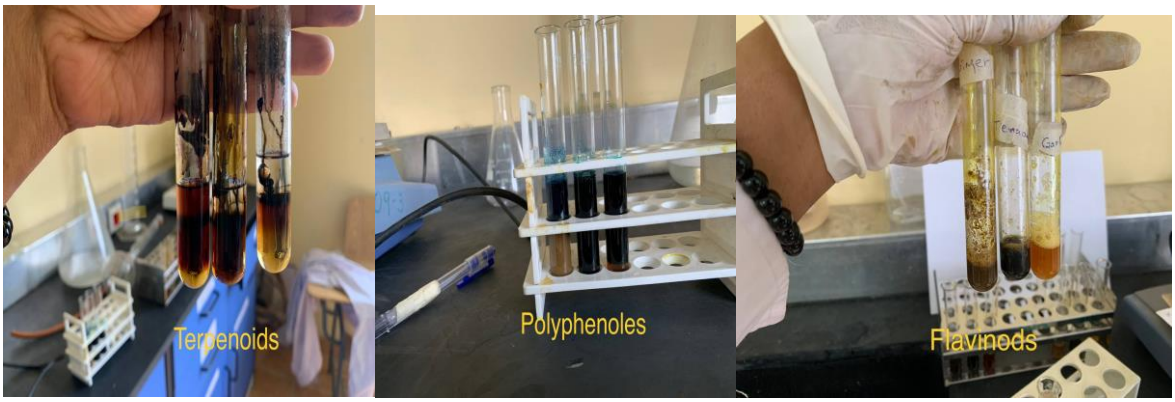


Appearance of Dead eggs inside shell post-administration of test extracts.

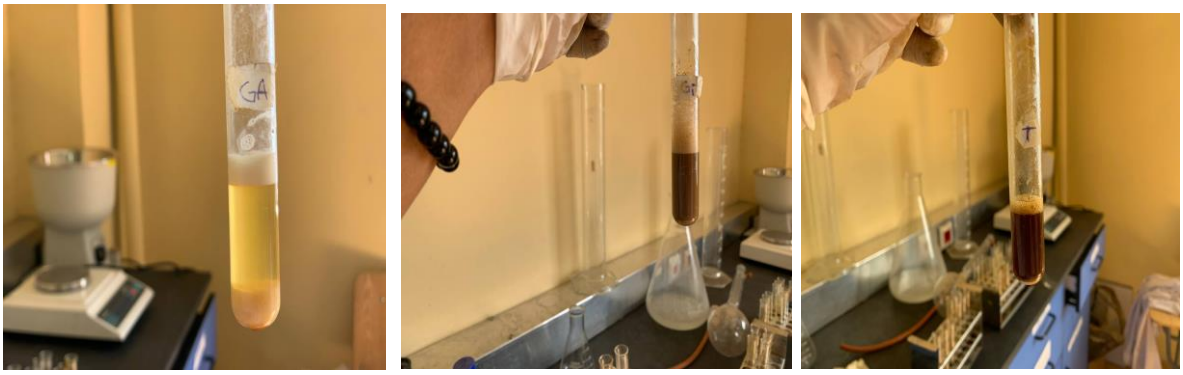


Appearance of dead L3 under microscope

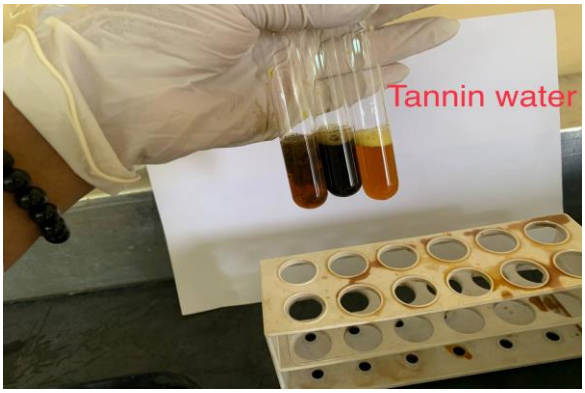
Annex 7: Phytochemical screening of the plant extracts



Pictures showing some phytochemical screening of the three plant extracts



Saponin Postive left (*Garlic*), middle (*Ginger*), Right (Tena Adam)



Annex 8: *In vitro* efficacy test recording format

Name of the plant used	**Concentration of methanol and aqueous extract `**	**Replications**	**No of dead worms in %**			
			0Hr	**3Hr**	**6Hr**	**9Hr**
Zingiber officinale (*Ginger*)	100mg/ml					
Ruta graveolens (Rue)						
Allium sativum (*Garlic*)						
Negative control 1 (DMSO)	50mg/ml					
Negative control 2 (PBS)						
Positive control ALB						
	25mg/ml					
	12.5mg/ml					

Ethical clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/07/02/14/2022

Name of Applicant: **Yodit Ayalew Sisay (DVM, MSc fellow)**

Address: Department of Pathology and Parasitology, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *In vitro anthelmintic activities of aqueous and methanolic extracts of Ruta graveolens, Allium sativum and Zingiber officinale against Haemonchus contortus*

Date of application:	**December, 2021**
Nature of the project:	**In vitro**
Target animal species:	**None**
Number of animals involved:	**None**
Study area:	Bishoftu, **Ethiopia**

Minutes No. and date of review: **VM/ERC/02/14/022, 01/03/2022**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

Democratic Republic of Ethiopia

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Please quote Our Ref. No. When replying
ፋክስ Fax 251-11-4339933 ስልክ Tel. +251 114338450 ፖ.ሣ.ቁ P.o.x. Box}34 ቢሾፍቱ፣ ኢትዮጵያ Bishoftu, Ethiopia