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ADDIS ABABA UNIVERSITY
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EFFICACY OF ENTOMOPATHOGENIC FUNGI AGAINST *AMBLYOMMA VARIEGATUM* TICKS AND THEIR CONTROL PRACTICES IN AND AROUND BISHOFTU, CENTRAL OROMIA, ETHIOPIA

MSc THESIS

BY

DEREJE TSEGAYE

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EFFICACY OF ENTOMOPATHOGENIC FUNGI AGAINST *AMBLYOMMA VARIEGATUM* TICKS AND THEIR CONTROL PRACTICES IN AND AROUND BISHOFTU, CENTRAL OROMIA, ETHIOPIA

A thesis Submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for the degree of Master of Science inVeterinary Parasitology

BY

DEREJE TSEGAYE

Department of Pathology and Parasitology

Advisor: Bersissa Kumsa (DVM, MSc, PhD, Professor)

JUNE, 2024

BISHOFTU, ETHIOPIA

Signature Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture Department of Pathology and Parasitology

Title **:Efficacy of Entomopathogenic fungi against *Amblyomma Variegatum* Ticks and their control practices in and around Bishoftu, Central Oromia, Ethiopia**

Submitted by: Dereje Tsegaye _____ _____

Name of student signature date

Approved for submittal to MSc thesis research assessment committee

1. Prof. Bersissa Kumsa	_____	_____
Advisor and Head of Post Graduate	Signature	Date
Prof. Getachew Terefe	_____	_____
Chairperson of Department	Signature	Date

Addis Ababa University

College of Veterinary Medicine and Agriculture Department of Pathology and Parasitology

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Dereje Tsegaye titled: **Efficacy of Entomopathogenic fungi against *Amblyomma Variegatum* Ticks and their control practices in and around Bishoftu, Central Oromia, Ethiopia,** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary parasitology.

1.Dr._____	_____	_____
Chairman	Signature	Date
2. Dr._____	_____	_____
External Examiner	Signiture	Date
3. Dr._____	_____	_____
Internal Examiner	Signature	date
4.Professor Bersissa Kumsa	_____	_____
Advisor	Signature	Date
5. Head of Department	_____	_____
	Signiture	Date

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Name: Dereje Tsegaye Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

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LIST OF ABBREVIATIONS

AChE	Acetyl Choline Esterase
AIT	Adult Immersion Test
ANOVA	Analysis Of Variance
CAHW	Community-Based Animal Health Workers
CI	Confidence Interval
CSA	Central Statistical Agency
EPF	Entomopathogenic Fungi
GABA	Gama Amino Butyric Acid
GPS	Global Positioning System
IPM	Integrated Pest Managements
LC	Lethal Concentration
OR	Odds Ratio
PDA	Potato Dextrose Agar
SDA	Sabouraud Dextrose Agar
SE	Standard Error
TBD	Tick Born Disease
USD	United States Dollar
UV	UltraViolet

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ABSTRACT

Ticks impact the health of animals and humans and are associated with numerous public health and economic problems around the world. The use of chemical acaricides has been the most commonly used method for tick control on livestock in recent years. However, chemical control of ticks has several disadvantages including high expense, lead to toxic residues in meat and milk, lead to acaricidal resistance and cause environmental pollution. The present in vitro experimental study on the efficacy of entomopathogenic fungi against adult *Amblyomma variegatum* collected from cattle was conducted from September 2023 to April 2024 in Bishoftu town. The entomopathogenic fungi were isolated from the soil samples in different parts central of Ethiopia. Furthermore, the knowledge, attitudes and practices of the animal owners regarding ticks and tick control was assessed through a structured questionnaire survey. Of the total of 80 soil samples examined for the presence of Entomopathogenic fungi, 17 samples from Bishoftu and Burayu were positive of which *Metarhizium anisopliae* were detected in 10 soil samples while the rest 7 soil samples were positive for *Beauveria bassiana.*The working concentrations were prepared from selected positive isolations at three strength levels ($1x10^6$, $1x10^7$ and $1x10^8$) conidia/ml was applied against *Amblyomma variegatum* while distilled water and Amitraz 12.5% were used as a negative and positive control, respectively. After applying different concentrations separately, and a mortality rate of 93% and 97.5% was recorded at the concentrations $1x10^7$ and $1x10^8$ conidia/ml respectively for *metarhizium anisoplie* and 93.33% and 97% for *beauveria issiana* after 15 days, with the lowest concentration ($1x10^6$) conidia/ml showing the lowest death percentage (83.25%) for *metarhizium anisoplie* and 80.7% for *beauveria issiana* whereas 76.75% mortality was recorded in the positive control Amitraz. The highest efficacy of Entomopathogenic fungi was observed for the highest concentration and time in increasing manner. The LC_{50} of 1×10^4 (% 95 CI = 1×10^3 - 1×10^8) conidia/ml for *Metarhizium anisopliae* and 1×10^4 (% 95 CL = 1×10^3–1×10^6) conidia/ml for *Beauveria bassiana,* and LC_{99} of $1x10^9$ (% 95 = $1x10^8$-$1x10^{13}$) conidia/ml for *Metarhizium anisopliae* and $1x10^9$ (%95=$1x10^7$-$1x10^{11}$) conidia/ml for *Beauveria bassiana* were recorded using probit regression analysis. This suggests that the EPF have great potential as the alternative approach to chemical acaricides for the control of ticks in Ethiopia.

Key words: *Amblyomma variegatum, Bishoftu, Burayu Entomopathogenic fungi*

1. INTRODUCTION

Livestock farming is one of the largest components of the agricultural sector in Ethiopia, which contributes to the national socio-economy (Gashaw *et al.,* 2014). The total gross value of livestock production is estimated at 74.338 billion birr, which takes 47.7% of the country's agricultural GDP and 16.5% of total GDP and 5–17% of total exports (Behnke, 2010). The socio-economic well-being of Ethiopians is strongly influenced by the ownership of cattle, which provide products such as milk and meat for many farmers as well as the energy needed to cultivate their land. Hides and skins are important livestock goods that generate significant export revenue (Gashaw *et al.,* 2014). The negative impact of external parasites including ticks and tick-borne diseases (TBDs) has resulted for the low level of projected potential obtained from the total contribution of livestock farming to food production, rural income and export earnings from livestock farming (Peter *et al.,* 2005).

Ticks are blood-sucking ectoparasites that are classified under the three families Argasidae, Ixodidae, and Nuttalliellidae under the phylum Arthropoda (Anderson, 2008). Of the three families, the argasid and ixodid ticks are of greater veterinary and medical importance. However, more efforts have been devoted to the control of ixodid than to argasid ticks. This can mainly be attributed to the differences in feeding habits and, behaviours. Additionally, in comparison to the two- or three-host ixodid ticks, the emergence of acaricide resistance is likely to be higher against one-host ixodid ticks (Walker *et al.,* 2003). Three-host ticks (*Amblyomma variegatum, Rhipicephalus appendiculatus*) are those that feed on a different vertebrate host at every stage of the life cycle, whereas one-host ixodid ticks (*Rhipicephalus decoloratus*, *Rhipicephalus annulatus*, *Rhipicephalus microplus*) feed on one host throughout their life cycle. Today, ticks and tick-borne diseases are a major problem for cattle production. They are widespread around the world, especially in tropical and subtropical countries and affect an estimated 80% of the world's cattle (Oluwoch *et al.,* 2009).

Economically, the most important ixodid ticks of cattle in tropical regions belong to the genera Amblyomma, Hyalomma, Rhipicephalus and subgenus Boophilus, (Lefebvre *et al.,* 2010). Of the 47 tick species reported from Ethiopia, most have been implicated as vectors of multiple diseases (Mekonnen *et al.,* 2001; Kumsa *et al.,* 2015, 2016). *Amblyomma*

variegatum (A.variegatum), *A.gemma, A. cohaerens, Rhipicephalus (Boophilus) decoloratus* and *Rh. pulchellus* are among the most important ixodid ticks (Mekonnen *et al.,* 2001; Kumsa *et al.,* 2016). *Rh. evertsi*, *Rh. praetextatus, Rhipicephalus pravus*, *Rh. muhasmae and Rh. bergeoni* have also been described (Mekonnen *et al.,* 2001 Kumsa *et al.,* 2016). *Hyalomma dromedarii*, *Hy. truncatum, Hy. rufipes, Hy. excavatum and Hy. impelatum* have also been identified (Walker *et al.,* 2003; Kumsa *et al.,* 2016).

Application of acaricides is the most widely used method of control of tick infestation in cattle. The lifespan of these chemicals has been shortened due to improper use of these compounds, which has led to the development of tick resistance to a number of commercial insecticides. Additionally, problems caused by chemical residues discovered in milk, meat, and the environment has prompted many to think about the necessity for better monitoring of their use (Almazán *et al.,* 2012). The application of acaricides is carried out using outdated, labor- and time-intensive, and therefore ineffectual, procedures. So far up to 25 different brands of acaricides are available on the market, and they are highly expensive. Therefore, despite routine application of acaricides, the challenge of TTBDs still persists (Musinguzi *et al.,* 2018).

For these reasons, researchers have proposed numerous alternative methods for evaluation and adoption of control of ticks affecting livestock in order to design an adequate integrated control and prevention schemes. Recently, many entomopathogenic fungi (EPF) species that could serve as biological control agents of ticks have been discovered. Among which *Metarhizium anisopliae and Beauveria bassiana* are regarded as the most probable candidates which were described to possess exceedingly lethal to a range of tick species at various life stages using laboratory studies. Although EPF have been used widely for the control of agricultural and forest pests, the applicability of bio-control potentials of entomogenous fungi against tick vectors of human and animal diseases has the promising results (Samish *et al*., 2004).

These fungi are microbes that specifically infect and often kill insects and other anthropods (Ghany, 2015). They are host-specific and hence, are non-pathogenic to plants and leave no toxic residue in crops (Jiang *et al.*, 2020). Additionally, they are non-toxic to animals and humans and are environmentally friendly as compared to chemicals (Skinner *et al.*, 2014). Entomopathogenic fungi are found in a wide range of environmental conditions and can infect a wide range of insects (Jiang *et al.*, 2020). Entomopathogens have been used as

control agents for insect pests for over a century and appear to be a more promising agent for the control of ticks than other potential biological control agents (Stafford and Allan, 2014). These fungi are present within the natural insect population and are considered as effective microbial control agents in integrated pest management (Jiang *et al.*, 2020).

Entomopathogenic fungi enter the host through the cuticle or natural openings on the host's body where they multiply and feed on the host's internal content causing the death of the host either by nutritional deficiency, tissue destruction, and disruption of normal biological functions or toxic substances from the fungus (Narladkar, 2018). The use of EPF will contribute to the reduction of the use of chemical products because they are safe for humans and other non-target organisms due to their high specificity to target organisms. EPF also reduce chemical residues in foods, are cheap and have high efficacy (Ghany, 2015).

The fungal species *Metarhizium anisoplie* and *Beauveria bassiana* have been reported to exhibit high virulence and hence, more widely used for the control of ticks worldwide (UB and Narladkar,2018). *Metarhizium anisoplie* and *Beauveria bassiana* have been tested for the control of ticks in very few African and South American countries (Ekesi and Maniania, 2002). Investigations have been undertaken on the pathogenicity of EPF on different tick stages such as adult, nymphs, larvae and eggs (Ghany, 2015; Kaaya and Hassan, 2000). However, there is little information regarding the EPF activity in African countries including Ethiopia. In Ethiopia EPF are not used for the control of ticks affecting domestic animals and thus their efficacy are not well known.

General objective

- To evaluate the efficacy of entomopathogenic fungi for the control of ticks collected from cattle.

Specific Objectives

- To isolate and identify entomopathogenic fungi from soil of the study areas.
- To evaluate the in vitro efficacy of entomopathogenic fungi against Amblyomma Variegatum tick.
- To assess the knowledge, attitude and practices (KAP) of farmers and animal owners of the study areas about ticks and tick-borne disease control

2. LITERATURE REVIEW

2. 1. Classification of Ticks

According to Torr *et al*. (2003), ticks belong to a group of animals known as the phylum Arthropoda, class Arachnida, subclass Acari, and order Parasitiformes. Ticks are Parasitiformes creatures belonging to the suborder Ixodida. The rare family Nuttalliellidae, which includes only one African species, and the two major families of the Ixodoidea Argasidae (soft ticks) and Ixodidae (hard ticks) make up this suborder (Rodríguez *et al.,* 2004).

There are about 683 species in the family Ixodidae, which is known as hard ticks. Ixodids show notable sexual dimorphism in adulthood. In males, the scutum covers the entire dorsum, but in females and immatures, it is reduced to a little podonotal shield behind the capitulum, allowing the idiosomal integument to be greatly distended while feeding. Ticks in the Ixodidae family, which includes thirteen genera, are rather large. Seven of these genera Amblyomma, subgenus Rhi. (Boophilus), Rhipicephalus, Haemaphysalis, Hyalom ma, Dermacentor, and Ixodes contain species that are significant for veterinary and medical use (Lora, 2001).The family Argasidae, or soft ticks, consists of about 185 species worldwide and have one important genus that infests cattle, Ornithodoros Adult argasids lack a dorsal sclerotized plate or scutum, their integument is leathery and wrinkled, their mouthparts are not visible from above, and they show no obvious sexual dimorphism. Argasidae are wandering ticks, which only remain on their host while feeding (Latif and Walker, 2004).

2. 2. Life Cycle of Ticks

Hard ticks mate on their hosts, with the possible exception of Ixodes, which may mate while the ticks are still attached to the plants. While feeding, male ticks will stay attached to their host and try to mate with multiple females. They give the female a spermatheca, or sac of sperm. The females mate just once, just as they are prepared to swell to full blood engorgement. They separate from the host when they have enough sperm saved to fertilize all of their eggs when they eventually engorge. In a single batch, female hard ticks can deposit between 2000 and 20,000 eggsArgasid tick females repeatedly deposit tiny batches

of eggs. All tick species deposit their eggs outdoors, never on their hosts (Charles and Robinson, 2006).

Ixodidae family members can have one, two, or three hosts during their life cycles. Ticks that follow the one-host life cycle spend all of their life on the same host from larval to nymphal to adult and only leave before producing eggs. The tick molts from larva to nymph on the first host in its two-host life cycle, although it will depart the host between the nymphal and adult stages. The second host could be a different species, an individual, or even the same host as the first. Most ticks that are significant for public health follow the three-host life cycle. The three hosts aren't usually the same species; depending on the tick's availability of a host, they might even be the same individual. Ticks classified as argasids go through two or more nymphal phases, each of which needs blood from a host. In contrast to Ixodidae ticks, which can remain attached to their hosts for several days during feeding, argasid ticks are designed to feed rapidly roughly an hour and then quickly depart from their hosts Walker *et al.,* 2003).

Ticks feed on their host at every step of their life cycle are parasites. They pierce the skin with their cutting mouthparts during eating, and they frequently utilize salivary cement an adhesive for adhesion. The blood and lymph secreted into this lesion provide food for the ticks. In reaction to respiratory products, all ticks move themselves toward possible hosts (Torres, 2008)

2. 3. The Distribution of Ticks in Ethiopia

In Ethiopia, there are wide regional variations in the distribution and number of tick species that infest household ruminants. Early in the 19th century, research on Ethiopia's tick fauna started. Since then, various domestic and international studies have identified the tick and TBD patterns; ticks are prevalent throughout the nation's agro-ecological zones (Desalegn *et al.,* 2015).

According to a study conducted in Ethiopia, one or more tick species affected 75.7% of cattle at 50% anticipated prevalence. Amblyomma (43.7%), Rhipicephalus (Boophilus) (20%), Rhipicep alus (17.7%), and Hyalomma (9.6%) were the average prevalence of each genera. Additionally, the research revealed that older and poor body condition animals had a higher frequency of tick infestations (Jelalu *et al.,* 2016; Asefa *et al.,* 2017).Tick

infestation prevalence is influenced by a number of variables, including breed, age, sex, and physical condition. Crossbreds have a higher frequency of tick-infected cattle than native breeds (Hordofa *et al.,* 2021). According to Walker *et al.* (2003) and Sileshi *et al.* (2007), the main tick genera present in Ethiopia's cattle population include Amblyomma, Rhipicephalus (Boophilus), Rhipicephalus, and Hyalomma. In several regions of the nation, the genus Ambylomma and Rhipicephalus (Boophilus) are predominant; Hyalomma and the subgenus Rhipicephalus also play a major role in cattle infestations (Solomon *et al.,* 2001).

2. 4. Economic Impacts of Ticks

Ethiopia is considered to have the highest number of cattle in Africa According to (CSA, 2022), the cattle industry is a significant contributor to the nation's economy and holds great potential for future growth. Due to its ability to provide milk, meat, skin, dung, and traction force, it is quite beneficial to smallholder farmers with limited resources (Mesfin and Lemma, 2001). Moreover, through the investigation of live animals, meat, hides, and skins, animal husbandry offers a boost to the country's economy, especially in terms of foreign exchange gain (*MoARD,* 2008). However, disease-related low animal health and production have significantly emerged as the main obstacle to the livestock industry's potential (Mekonnen *et al.,* 2001).

In particular, a wide range of intercellular bacteria, including Ehrlichia, Anaplasma, and Rickettsia, are transmitted by ticks. Similarly, ticks are the primary vector for the transmission of various piroplasm protozoa, including *B. bigemmina, T. annulata*, and *T. parva.* The most serious tick-borne illnesses, including heartwater, babesiosis, theileriosis, babesiosis, and East Coast Fever, are caused by them. Certain diseases, such as anaplasmosis, theileriosis, and babesiosis, are both benign and deadly (Jongejan and Uilenberg, 2004).

The blood and/or lymphatic systems are frequently affected by these illnesses, which can result in fever, anemia, jaundice, anorexia, weight loss, decreased milk production, swollen lymph nodes, dyspnea, diarrhea, neurological abnormalities, and even death (Nejash, 2016). Animal mortality, decreased productivity and fertility, deterioration and rejection of hides and skins, and severe irritation and trauma can all result in significant financial losses for smallholder farms, the tanning industry, and the nation as a whole. Controlling ticks and

the spread of diseases carried by ticks is still a problem for animal husbandry globally, particularly in tropical and subtropical areas, because of the economic and veterinary significance of ticks (Lodos *et al.,* 2000).

The wide range of animal health issues is influenced by all of these variables. Around eighty percent of cattle worldwide were at danger for tick infestation and diseases carried by ticks, resulting in massive financial losses estimated to be between 13.9 and 18.7 billion USD yearly (Ghosh *et al.,* 2007). An estimated 700 million USD are spent globally each year on tick management and productivity losses (Yilma *et al.,* 2001). Tick infestation is thought to cause a conservative estimate of one million USD in losses each year in Ethiopia due to the devaluation of skin and hides. Furthermore, ticks can induce mild to severe inflammatory reactions that occasionally require teat surgery (Nejash, 2016). Ticks were responsible for about 65.5% of the primary flaws in hides, and alone eastern Ethiopia lost 500,000 USD as a result of hide and skin degradation (Bekele, 2002).

Furthermore, the predicted overall loss will be substantially higher than this when losses from decreased output, fatalities, and tick control expenses are included. There is a lack of comparable data regarding the economic damages caused by tick-borne diseases and ticks in African livestock. However, just East Coast Fever is thought to be responsible for 168 million USD throughout Eastern, Central, and Southern Africa. These losses are exacerbated in tropical and sub-tropical environments where the host is subjected to stresses associated with sub-optimal nutrition and high environmental temperature. In addition to this, the costs associated with maintaining chemical control of ticks in tropical and subtropical regions of the world have been estimated at 25.00 USD per head of cattle per year (Pegram, 2001).

2. 5. Methods of Tick Control

A thorough understanding of the ecology or epidemiology of ticks in particular climatic conditions is necessary for the effective implementation of sensible and long-lasting tick control strategies in the region where livestock is raised. However, the majority of the time, effective and trustworthy strategies for managing TBD and cattle ticks rely on the use of acaricides without a thorough understanding of the relevant ecology or epidemiology. Although the most effective and dependable single strategy for controlling ticks and their

hemoparasites is the use of acaricide, complementary ways have been developed and are being explored to enable integrated control tactics against ticks. (Alanr, 2011).

It is essential to evaluate the availability of each of these options as well as the advantages and disadvantages of each control program before selecting an action that targets the parasitic and free-living phases of the life cycle as well as the role that ticks play in the transmission of TBDs (Kirby, 2010). The tick control methods that are most frequently used in various parts of the country are briefly described below:

Genetic tick control: The use of acaricides to control ticks in the cattle population has its own drawback. Therefore, the production of various breeds of cattle with the potential to be genetically resistant to ticks can contribute to the creation of alternative methods of control to mitigate this issue. As a result, Bos indicus cattle are more resistant to ectoparasites than Bos taurus cattle. The crossing of these two breeds has also produced high-quality meat and increased resistance to the environment (Bianchin *et al*., 2007).

Biological tick control: The introduction of tick parasitic agents such as wasps, birds, parasitoids, Entomopathogenic nematodes, EPF, and bacteria is the primary focus of the biological tick control method (George *et al.,* 2008). In addition, by consuming a large number of ticks, predators such as rodents, birds, ants, spiders, lizards, and beetles play a significant role in tick control (Latif and Walker, 2004). The wasp lays eggs in the engorged ticks, eats the tick's larvae, which then emerge as an adult to attack another tick, and parasites (Nematodes and fungi) attack the ticks' soil-living stages (Kirby, 2010).

Ecological tick control: The treatment of host-linked disease and habitat uses an ecological control strategy. Removing vegetation that serves as a tick haven is necessary to modify the plant cover and control tick populations in the ecosystem (Kirby, 2010). The tick burden has been reported to have decreased as a result of the application of pasture management and seasonal modifications in cattle grazing regions (Walker *et al.,* 2003).

Chemical-based tick control: During times of high tick infestation, acaricide treatments are frequently applied in a suppressive manner, applying multiple treatments on a regular basis. In the short term, suppressive treatments are the most effective; keeping animals nearly tick-free, thereby lowering the likelihood of disease transmission and the direct

effects of ticks. However, this method will disproportionately target ticks with acaricide resistance (George *et al*., 2008).

An ideal acaricide would be inexpensive, easy to apply, have a strong knockdown effect, be safe for humans and livestock, and have enough residual effect on female ticks to stop them from laying eggs and keep cattle from being re-infested by larvae and leave no traces in milk or meat. It should have a sharp cut-off in efficacy with time and not select for resistance through a prolonged, gradual decay on the animal. Inappropriately, no such ideal acaricide has yet been developed. Tick resistance to acaricides is a growing issue and a real economic threat to livestock and the use of acaricides for tick control generally has limitations. Besides, livestock owners rely solely on acaricides to control ticks. However, they do not have access to guidelines on how to profit from ticks or how to identify and address issues with resistance to acaricides (Nejash, 2016).

2. 6. Development of Acaricides Resistance in ticks

Resistance is generally first recognized as the failure of a drug to control parasitism, but the formal definition of resistance is a shift in the target species' susceptibility to a drug (Corley *et al*., 2013). The ability of a parasite strain to live or to reproduce despite the administration and absorption of a medicine given in dosages equal to or greater than those typically indicated is defined as resistance by the scientific group of the World Health Organization (Dzemo,2022). Acaricidal resistance comes in three different forms. According to Abbas *et al.* (2014), these are acquired resistance, cross-resistance, and multiple resistances.

Acquired resistance refers to resistance that inherits the decreasing activity of drugs over time (Meyer *et al*., 2012). A decrease in the population of ticks that are susceptible to an acaricide is accompanied by an increase in resistant strains due to a direct relationship between the concentration of the drug, continued exposure, and degree of acaricide resistance (Faza *et al*., 2013).

Cross-resistance is the sharing of resistance among different acaricides with a similar mode of action, like two organophosphates groups (coumaphos and diazinon) and one carbamate (carbaryl) acaricides in several strains of *R. microplus* (Abbas *et al*., 2014). These two acaricides (organophosphates and carbamates) exert their toxic effects on ticks by

inhibiting acetylcholinesterase (AChE), which gives function to the nervous system of invertebrates (Jensen *et al*., 2011). The resistance of ticks against carbamates and organophosphates is developed by reducing sensitivity to a mechanism of action of AChE (Dawkar *et al*., 2013).

Multiple resistances are resistance to more than one therapeutic agent, even if they have different modes of action. Nowadays, it is a threat to the chemical control of cattle ticks. In the tick population, multiple resistances against acaricides with different modes of action lead to suspect that resistance may be metabolic (Bielza *et al*., 2007).

2.7. Mechanism of Acaricides Resistance

The first development of resistance of *B. microplus* and *B. decoloratus* to arsenic was reported from Australia and South Africa respectively and reports of amitraz resistance to Boophilus spp in different parts of the world. The emergence of resistance to a variety of acaricides like organophosphate, pyrethroid, formamidine, and macrocyclic lactone acaricides in both single and multi-host ticks was an indication of the gradual development of resistance (George *et al*., 2004).

In addition to this, macrocyclic lactone resistance of *B. microplus* was reported in Brazil, especially to doramectin with cross resistance of ivermectin. Widespread use of arsenic, chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids, and macrocyclic lactone for tick control has caused major concern for the development of resistance. Therefore, understanding basic knowledge of the mode of action of acaricides is very important to know the mechanisms involved in the development of resistance (Martins and Furlong, 2001).

2.7.1. Resistance against organochlorine

Organochlorine was the first synthetic insecticide to be marketed and has been formulated for the control of ticks on cattle since 1946 (Abbas *et al*., 2014). The mode of action of these compounds is binding at the picrotoxinin site in the γ aminobutyric acid (GABA) chloride ionophore complex (Hope *et al.,* 2010) which inhibits Cl flux into the nerve (Corley *et al*., 2012). The function of the organochlorine with GABA-ergic inhibitory neurons is impaired and hyperexcitation results in death. The mechanism of resistance was

preceded by boosting metabolism and reducing the absorption of the chemical (George *et al*., 2004; Abbas *et al*., 2014).

2.7.2. Resistance against amidines (Amitraz)

Amitraz is a member of the amidine class and has been used as an effective treatment against the ticks of cattle for more than 45 years, but resistance has been reported (Mendes *et al*., 2013; Abbas *et al*., 2014). The mode of action of amitraz is by inducing its toxic effects on a receptor for the neuromodulator (octopamine), which contains two nucleotide substitution molecules on the resistant strains of ticks that result in amino acids different from all the susceptible strains (Corley*et al*.,2013). In that, it is synergistically involved with P450 cytochrome monooxygenases by modification of the target site. The Discovery of these mutations in amitraz-resistant ticks provided the first evidence for the possibility of an altered target site as a mechanism of amitraz resistance in ticks. However, the exact mechanism of resistance to amitraz is not well described (Guerrero *et al*., 2012).

2.7.3. Resistance against pyrethrins (Pyrethroids)

Pyrethrins are naturally occurring compounds derived from flowering plants called the chrysanthemum family. They have a quick knock-down effect against arachnids, but they are unstable in the environment and do not persist actively long enough to kill ticks. Pyrethroids are the synthetic version of pyrethrins, which are designed to be more stable than the pyrethrums and to achieve a longer-lasting effect. Both pyrethrins and pyrethroids are potent neurotoxins. They act on sodium ion channels and thus cause nerve excitation due to changes in nerve membrane permeability to sodium and potassium ions (Weston *et al*., 2013; Abbas *et al*., 2014).

Voltage-gated sodium channels are the target of pyrethroid activity and resistance development in a wide range of pests and disease vectors (Guerrero *et al*., 2012). The involvement of p450s and esterases in pyrethroid resistance has been demonstrated for many species of ticks (Abbas *et al*., 2014) by developing a mutation that decreases the channel sensitivity to pyrethroids (Oliveira *et al*., 2013).

2.7.4. Resistance against organophosphates

The chronological order of acaricidal development started with arsenic, which was the first effective acaricide used for controlling ticks, and tick-borne diseases in different parts of the world before resistance developed. After resistance developed, this chemical was replaced by chlorinated hydrocarbons (DDT), which have been highly persistent in the environment and used extensively throughout the world for controlling ticks (Rajput *et al*., 2006).

Organophosphates (OPs) were introduced around 1950, to replace chlorinated hydrocarbons, which have a wide range of activities against ticks at very low concentrations and developed significant resistance. Among the first chemical groups used to control arachnids, OPs were the leading one. The mechanism of action of OPs was exerting their toxic effects on ticks by inhibiting acetylcholine esterase (AChE), which is a key enzyme to the functioning nervous system (Faza *et al*., 2013; Temeyer *et al*.,2013a,b).

When ticks are poisoned with a cholinesterase inhibitor, the cholinesterase is not available to help break down the acetylcholine, and the neurotransmitter continues to cause the neuron to "fire" or send its electrical charge. This results in overstimulation of the nervous system and the death of the arachnid. But, the mechanism of resistance for Ops can be developed by increasing the activity of AChE due to mutations in AChE genes and oxidative metabolism (Van Leeuwen *et al*., 2009; Lwande *et al*., 2012).

2.7.5. Resistance against macrocyclic lactones

Macrocyclic lactone acaricides include avermectins and milbemycins, which are naturally occurring products of actinomycetes (genus Streptomyces) by the process of fermentation. Milbemycin was first derived from a culture of Streptomyces hygroscopicus and is structurally similar to the avermectins but lacks the disaccharide at C13 and was first reported as the effective acaricidal agent by (Ferreira *et al.,* 2022).

It blocks the transmittance of electrical activity in nerves and muscle cells by stimulating the release and binding of gamma-aminobutyric acid (GABA) at nerve endings and causes an influx of chloride ions into the cells leading to hyper polarization and subsequent paralysis of the neuromuscular systems (Abbas *et al*., 2014). The mechanism of resistance

in nematodes against macro cyclic lactones is due to target site insensitivity of the GABA or glutamate-gated chloride ion channels (Lovis *et al*., 2013).

2. 8. Risk Factors in Resistance Development of Ticks

Populations of ticks, especially *B. microplus*, developed resistance to many classes of acaricide, including chlorinated hydrocarbons (DDT), pyrethroids, organophosphates, and formamidines (amitraz). This resistance development in the tick population is associated with three factors. First, the genetic factors of parasites include the dominance of resistance alleles, the number of genes involved, the genetic diversity of the population, the relative fitness of resistance, and genetic recombination (Abbas *et al*., 2014).

Second, biological aspects are mainly associated with the host–parasite relationship and mechanism of selection for resistance. For example, parasites that induce effective immunity in their hosts have weaker selection pressure for resistance because immunity selects parasites regardless of drug-resistance status, and this reduces the chance of parasites surviving and reproducing (Abdullah *et al*., 2012).

Third, operational factors include the chemical nature of the drug, drug persistence in the host, application and selection threshold, life stages selected, mode of application, frequency of treatment, frequent use of the same acaricide for a long period of time, under-dosing, and poor drug quality (Rezende *et al*., 2013). The use of acaricide greater than five treatments per season is a positive risk factor for acaricide resistance (Jonsson *et al*., 2000).

2. 9. Strategies to Minimize Acaricidal Resistance

A well-understanding of tick population dynamics and their survival in diverse natural habitats might be essential to initiate the formulation of integrated, suitable, efficient, and economic tick control measures (Luciana *et al*., 2011). Besides, information on the status and magnitude of acaricide resistance is vital in deciding the appropriate tick and tick-borne disease control strategy in different localities in Ethiopia (Gebre *et al*., 2004).

There are a few options used to reduce resistance, by diagnosing the evolution of acaricide-resistant tick, in that acaricides become ineffective in control programs (George *et al*., 2004). These include the rationale use of acaricides by means of monitoring, the use of combinations (mixtures) of acaricides (Lovis *et al*., 2013), using vaccination to enhance

immunity in cattle and improving nutritional management as well as improving genetic resistance in cattle (Ayres *et al*., 2013).

Besides, rotation or alternation of acaricides having different modes of action, environmental management like pasture burning, pasture alternation or rotation, house management, and development of effective diagnostic techniques are used as ways of tick resistance mitigation options (Abbas *et al*., 2014).

2. 10. Role of Entomopathogenic Fungi in Tick Control

The order Hypocreales of the Ascomycota is home to a large number of common and significant entomopathogenic fungi (EPF). These consist of the sexual (teleomorph) state Cordyceps and the asexual (anamorph) phases Beauveria, Isaria, Hirsutella, and Metarhizium. Any component of the kingdom that has the ability to infect insects and other terrestrial arthropods, including spiders, mites, and ticks, is referred to as an entomopathogen (Baron *et al.,* 2019). Due to their heterotrophic metabolism, they are forced to adopt a host-dependent lifestyle. They are classified as saprophytes, parasites, or symbionts based on whether their association is beneficial, harmful, or neutral for the host organism. The majority of our discussion will be on host-harming parasitic entomopathogen fungi (Bava *et al.,* 2022).

Although EPF have been used widely for the control of agricultural and forest pests, little effort has been made to evaluate the applicability of bio-control potentials of these fungi against ticks which are vectors of human and animal diseases. Among these fungi, *Beauveria bassiana* and *Metarhizium anisopliae* received major attention (UB and Narladkar, 2018). Different studies on the potential use of *M. anisopliae* and *B. bassiana* as entomopathogenic agents were performed in laboratories; however, there is an urgent need for checking the novel strains and even fungal species from different geographic regions to find out potent species(Strausser *et al.,* 2000).

2.10.1. Biology of the entomopathogenic fungi

This section highlights the biology of Beauveria fungi and Metarhizium. Both Beauveria and Metarhizium are classified as Deuteromycetes (Schulte *et al.,* 2004). The morphological group of fungi known as Hyphomycetes is found within the Class

Deuteromycetes. These are filamentous fungi that reproduce by conidia that are typically formed aerially on conidiophores arising from the substrate. Conidia attach to the cuticle, germinate, and penetrate the cuticle; once inside the hemocoel, the mycelium spreads throughout the host, forming hyphal bodies known as blastospores. The external integument is the most common site of host invasion, though infection through the digestive tract is also possible. A combination of fungal toxins, physical blood circulation restriction, nutrient loss, and/or organ invasion often results in insect death. Hyphae typically develop from the cadaver after the host dies away, and in the right abiotic conditions, conidia are generated on the host's exterior. After then, the wind or water scatters these (UB and Narladkar, 2018).

Metarhizium: Metarhizium Found all throughout the world and is one of the most prevalent EPF. The species mostly infects soil-dwelling insects and is soil-borne. According to UB and Narladkar (2018), *Metarhizium anisopliae* has a wide host range that includes arachnids and five orders of insects with more than 200 species. On terrestrial insects, the life cycle starts with a conidium adhering to the host's cuticle to form an appressorium, then a penetration peg entering the cuticle. Once inside the hemocoel, hyphae form and release toxins that kill the host 4–16 days (mainly depending on the species) after contamination (Strausser *et al.,* 2000). Histopathological analyses of elaterid tissues infected with *Metarhizium anisopliae* indicate that toxins, or destruxins, cause the host to die by causing the tissues to degenerate because their membranes are no longer structurally intact, which leads to the cells becoming dehydrated from fluid loss. *Metarhizium anisopliae* has demonstrated promise as a mosquito control agent in numerous laboratory tests (UB and Narladkar, 2018).

For conidia to germinate, a relative humidity of at least 92% is often needed. According to Morley *et al.* (1996), conidia formed in paraffin oil had lower germination rates (from 93% to 73 days) than conidia held under dry circumstances, which have greater germination rates initially (96%, declining to 80% after 60 days). The conditions that allow conidia to survive the longest are either low temperature and low RH (40 0C and 0% RH) or moderate temperature and high RH (26 0C and 97% RH or 19 0C and 97% RH). The substrate on which conidia are formed is not as important to spore survival and virulence as storage conditions are. The fungus is easily cultured in vitro. This fungus is a very promising control agent because it does not germinate in the tick environment until it is

actually exposed to a host, which leads to its persistence in the environment. Moreover, unlike *Beauveria bassiana*, its effect is not restricted to times when the host is molting (UB and Narladkar, 2018).

Beauveria: With a worldwide distribution, Beauveria is one of the most commonly isolated EPF genera. When *Beauveria bassiana* conidia are dusted into the water surface of breeding locations, they effectively destroy mosquito larvae. Since conidia are hydrophobic, they float on the water's surface and come into contact with mosquito larvae at the siphon's tip, which is where they feed below the surface. According to Goette *et al*. (2008), one of the most important variables influencing the results of *Beauveria bassiana* tests conducted in the field and in the lab is humidity. Temperature doesn't seem to have an impact on infection. However, high temperatures may be detrimental to conidia, particularly when combined with high humidity levels Conidia and blastoconidia are the phases of the fungus that are most effective against larvae, with the latter stage being significantly more harmful. One drawback of employing conidia is that they don't leave a residue. Although blastoconidia are very easy to cultivate, the challenges associated with keeping this type of conidium have led to the abandonment of its production (Strasser *et al.,* 2000).

***Verticillium lecanii*:** Previously recognized as a single species, fungi in the genus Lecanicillium are significant insect diseases, and some of them have been turned into commercially available biopesticides. Certain isolates have activity against fungus or phytoparasitic nematodes as well. Lecanicillium species directly pierce the fungal plant pathogen's cell wall and the insect integument by means of hydrolytic enzymes and mechanical forces. The mode of action is connected to the plant pathogen's mycoparasitism as well as the colonization of host plant tissues, which causes pests bassiana to develop an induced systemic resistance (Goette *et al.,* 2008). Arthropods, nematodes, plants, and fungi were among the many different species with a wide range of host ranges that belonged to the same genus Verticillium until recently (Zare and Gams, 2001). Lecanicillium species have been isolated from a wide range of insect orders and have a broad host range. Lecanicillium spp (de Faria and Wraight, 2007).

In general, follow the typical pathogenesis pathway of entomopathogenic mitosporic fungi: conidia adhering to the host cuticle, germination occurring within the cuticle, penetration of the cuticle, production of blastospores within the hemocoel, mycelia ramifying and

invading tissues, resulting in the death of the host, and finally, conidia producing on the cadaver's surface. *Lecanicillium muscarium* is marketed as a biopesticide and has a wider host range. It has been isolated from a variety of substrates, primarily fungi and insects. It has been commercialized as the bio-pesticides (de Faria and Wraight, 2007).

2. 10. 2. Source and availability of Entomopathogenic fungi

Because soil is shielded from ultraviolet (UV) radiation and other harmful abiotic and biotic factors, it is thought to be a suitable environmental home for EPF. Until it adheres to a suitable host in the surrounding microenvironment or forms an endophytic association with plants, the fungus lives in the soil as saprotrophic mycelia or dormant propagules (Domsch *et al*., 1980).

Soil is a frequent habitat for the fungal entomopathogens belonging to the genera Beauveria, Conidiobolus, Metarhizium, and Isaria (Paecilomyces). It's possible that these fungi can live in soil as saprophytes. Additionally, EPF can be found in a variety of environments, such as urban, desert, grassland, and aquatic forests. Metarhizium and Beauveria are endophytes of plant roots, stems, and leaves that develop intricate connections with plants. (Jaber and Enkerli 2017).

The most frequent fungi discovered in soils from organic fields are *Beauveria bassiana*, Lecanicillium sp., Isaria fumosorosea, and *Metarhizium anisopliae*; soils from conventional fields are more likely to include *I. fumosorosea* (Tkaczuk *et al.,* 2014). It is also possible to separate the fungal species from insects that inhabit different environments. For instance, it has been found that some insect host species are naturally infected by *Beauveria bassiana*, *Nomuraea rileyi*, *Paecilomyces fumosoroseus,* and *Paecilomyces farinosus* (Thakur and Sandhu, 2010).

There are commercially produced bio-insecticides based on pathogenic fungi on the market (Hafiza *et al.,*2014).For the purpose of managing crop pests, several commercial formulations of EPF have been created. Almost 700 species and 90 genera, or nearly all of the major fungal classes, have been identified as insect-infecting fungus in recent times. These EPF species have a great deal of potential as biocontrol agents because they cause fungal infections in insect populations when they are released into the environment (Pucheta *et al*., 2006, Ramanujam *et al*., 2014).

2.10 .3. Methods for isolation of entomopathogenic fungi from soil samples

Selective media: The soil environment is home to a diverse array of fungi, each of which serves a different ecological purpose. Numerous bacteria and the majority of these fungi can grow in vitro on artificial medium. These properties have long been used to separate microorganisms from soil samples, and particular media have been created to target particular microbe groups. Additionally, several media have been created for the specific isolation of fungi that are harmful to insects. Broad-spectrum antibiotics, such as tetracycline, streptomycin, or chloramphenicol, can suppress the growth of bacteria. The primary remaining obstacle to applying this isolation method is the slower growth rate of the hypocrealean entomopathogenic fungi in contrast to the saprotrophic fungi that are opportunistic and commonly present in the soil environment. Substances that stop these fungi from outgrowing the target species must therefore be included in media content. In general, the most studied species are *Metarhizium anisopliae, Beauveria bassiana,* and *B. brongniartii* (Correa *et al.,* 2022).

Insect bait method: The saprotrophic properties of hypocrealean entomopathogenic fungi are exploited through the use of selective media. Insect baiting is one way to take use of the fungi's capacity to infect a host, though. Originally designed to separate entomopathogenic nematodes from soil samples, Zimmermann (1986) also reported that fungus might occasionally be isolated using this technique. This approach might also be a standard method for isolating EPF.Insects, wich are easily reared and susceptible to the fungi, must be utilized for the process to work. Traditionally, mealworm larvae (*Tenebrio molitor*, Coleoptera: Tenebrionidae) or the extremely sensitive wax moth larvae (*Galleria mellonella,* Lepidoptera: Pyralidae) have been used as bait insects. A common method for searching for native species of entomopathogenic fungi is to bait soil samples with *G. mellonella* larvae (Vanninen *et al.,* 1989; Vänninen 1996; Chandler *et al.,* 1997; Bidochka *et al.,* 1998; Klingen *et al.,* 2002; Keller *et al.,* 2003; Meyling and Eilenberg, 2006b)

2. 10. 4. Mode of action of Entomopathogenic in ticks

On adult ticks: The crucial moments in the interaction between EPF and their arthropod hosts are the conidial germination and appressoria production. The lipid content of the tick epicuticle specifically impacts the germination of EPF conidia that causes the tick mortality. Conidia formation, adhesion, and germination are acknowledged as the primary

virulence factors against arthropods. Given that this kind of mode of action is seen against all stages; egg, larva, nymph, and adult fungus seems to be a highly effective anti-tick agent (de Faria and Wraight, 2007). Fungi quickly infiltrate internal organs when germ tubes have penetrated the cuticle of the arthropod, and kill the host arthropod. Mycotoxins, which are also released by these fungi, may potentially contribute to death (UB and Narladkar, 2018).

Several cuticle-degrading hydrolytic extracellular enzymes, including chitinases and B-1, 3-glucanases, are produced by different strains of *B. bassiana* and are thought to be indicators of fungal pathogenicity. During the course of the infection, these fungi release harmful compounds that aid in the development and spread of the illness. In addition*, B. amorpha* and *B. bassiana* generate chitinases and subtilisin-like proteases when ticks are present (Campos *et al.,* 2005).

Hydraulic enzymes working in concert to break down apressorium and produce mechanical pressure Deuteromycete *M. anisopliae* has a wide host range and was initially identified in 1880 as a possible biological control agent for agricultural pets. Because apressorium forms and is broken down by the combined activity of hydrolytic enzymes like proteases, chitinases, and lipases, the fungus actively invades the hosts through the cuticle by mechanical pressure (Gindin *et al.,* 2014).

On eggs of ticks*:* Tick surface lipids, specifically pentane and DCM, are important for the germination of conidia and the development of fungal strain appressoria. Thus, the only way that fungi affect tick eggs of a certain species is through interacting with the lipids that are present on the surface of the tick egg and conidial density. Compared to the other two species of ticks, the lipids on *Rh. sanguineus* eggs induced the germination of more conidia within 12 hours of contact, suggesting that the fungal strains that affect tick eggs differ in their sensitivity (Ment *et al.,* 2010)

Egg wax and tick egg age: Tick egg susceptibility to *M. anisopliae* was also noted based on a single component, namely egg age. Changes in the egg cuticular chemicals brought on by oxidation may be the reason for the decrease in egg resistance to the fungus (Gindin *et al.,* 2014). The female tick secretes a waxy covering onto each egg when it is laid by Gene's organ.This coating has fungicidal properties in addition to making the eggs waterproof. After secretion, the wax hardens, increasing its melting point, making it

stickier and more capable of supporting the egg mass. Metarhizium; as the egg ages, its surface wax oxidizes more quickly and its tegument's content of unsaturated fatty acids decreases, decreasing the egg's antifungal qualities. Tick eggs therefore seem to be more vulnerable to the fungus than adult ticks (Greesma, 2017). In engorged female ticks, fungal infection frequently led to longer times for pre-oviposition, oviposition time, egg incubation, and egg hatching of the egg mass, along with decreased egg production. (Gindin *et al.,* 2001).

3. MATERIALS AND METHODS

3. 1. Description of the Study and sampling areas

The study was conducted from November 2023 to April 2024 in and around Bishoftu town in central Oromia, Ethiopia. The capital of the study area, Bishoftu, is located about 47 km southeast of Addis Ababa, on the slope of the Great Rift Valley and the geography of the area is characterized by creator lakes (Fig 1). It is located at 9°36\" N latitude and 9°36\" E longitude and at an altitude of 1850 meters above sea level in central Ethiopia. It has a human population of more than 200,000 people. It experiences a bimodal rainfall pattern with the main rainy season ranging from June to September during which 84% of the rain is expected and a short rainy season from March to May with an average annual rainfall of 800 mm. The average annual minimum and maximum temperatures are 12.3°C and 27.7°C, respectively, with an overall average of 18.7°C. The mean relative humidity is 61.3% (CSA, 2017).

The second study area, Burayu, is one of the six subcities of Shegar city Located in the Oromia Region. It serves as the headquarters of the Burayu Zone. It's situated approximately 15 km west of Addis Ababa and the town stretches from 90 01′ 00 ″ to 90 06′ 00 ″ N latitude and 38◦ 36′ 00″ to 38◦ 42′ 00″ E longitude The town has a population of 127921 according to the Central Statistics Agency (CSA,2023). The social structure of the town is cosmopolitan by composition where different ethnic groups make up varying percentages. According to CSA (2006) the majority (58.1%) are local Oromo followed by Amhara (21%) while two other ethnic groups make up about 15% of the population. The 2007 national census report found out that about 62% of the population residing in Burayu.

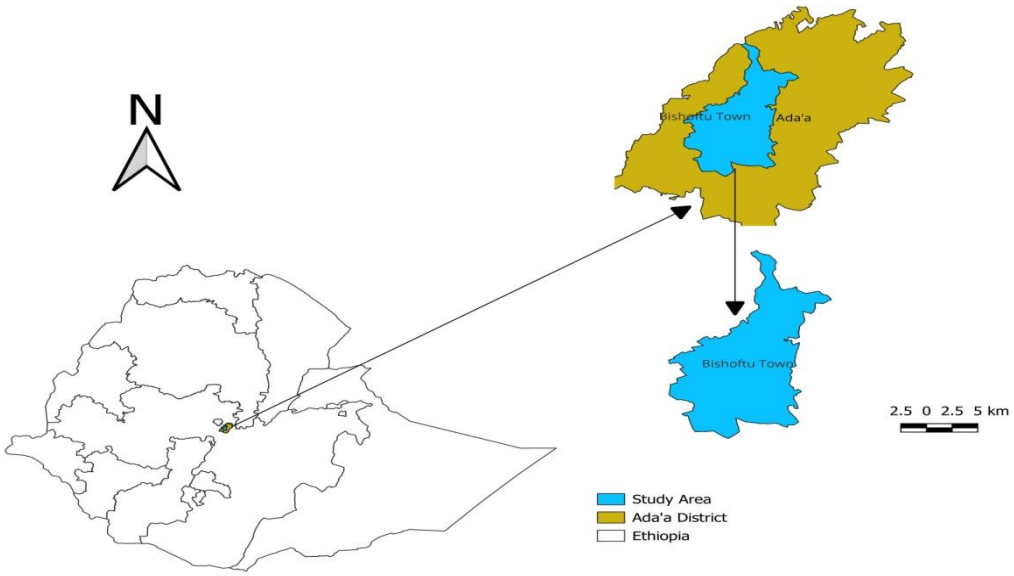

Figure 1: Map of Ethiopia showing the preset study area.

3. 2. Study Design

The study employed an experimental design and cross-sectional questionnaire survey to assess the knowledge, attitude and practices of livestock owners and farmers regarding tick control methods used in the study area. For experimental study, soil samples was collected from Bishoftu and Burayu agroecological locations where as *Amblyomma variegatum* tick was collected from Bishoftu and its surroundings. During the study period entomopathogenic fungi (EPF) were isolated from soil using selective media and insect baiting methods followed by evaluation of the in vitro efficacy of EPF on *Amblyomma variegatum* ticks. Furthermore, the study compared the efficiency of the methods of isolation of EPF from soil samples using insect baits with the method of artificial selective media usage. The use of *Galleria mellonell* (*Lepidoptera: Phyralidae*) as insect bait in EPF isolation was employed based on previously done by Correa *et al,* (2022).

3. 3. Questionnaire Survey

A semi-structured questionnaire survey was conducted involving farmers in and around Bishoftu town in central Ethiopia who own cattle, willingness to participate in the survey and had experience of using acaricides as the primary selection factors. All respondents were agreed to participate on the questionnaire regarding their KAP about the usage of acaricides and tick control practices in their area.

The respondent's knowledge and practical activities (good and poor) was tested and determined using the criteria including knowing season of heavy tick infestation, knowledge of tick sources, experience with the use of acariicides, awareness of tick resistance to chemicals, training in the use of acaricides, habit of purchasing chemicals from authorized sources and awareness of the potential harms of acaricides to animals, humans and the environment. Likewise, a positive attitude was attributed to respondents who expressed a greater willingness to participate in a tick eradication program and to use all available methods (ethno-veterinary practices and conventional acaricides) to control tick populations; reluctance to do so was categorized as a negative attitude. The data were coded by rating desirable answers as 1 and undesirable answers as 0 for a given question.

The final knowledge and practice assessment scores were dichotomized for further analyzing by logistic regression and those answered ≥ 50% correct were considered to have good knowledge and valued as 1, while those answered < 50% considered as poor and valued as 0. Likewise, those answered ≥ 50% positive attitude and valued as 1 and for those answered < 50% considered as negative attitude and valued as 0 according to (Alzahrani *et al.,* 2022).

The sample size for questionnaire survey was done based on the formula recommended by Arsham (2002).

$N=0.25/SE^2=0.25/ (0.05)^2=100$

Where N=sample size, SE=standard error assuming the standard error of 5% at a precision level of 0.05 and the confidence interval of 95%.

Accordingly, 100 volunteer individuals were thought to be interviewed, but to increase the precision, 180 (128 males and 52 females) were interviewed.

3. 4. Collection and Processing of Soil Samples for Fungi Isolation

3. 4. 1. Soil sample collection

During the study period a total of 80 soil samples were randomly collected from various agroecological zones in and around Bishoftu (40 samples) and Burayu (40 samples) towns for isolation of entomopathogenic fungi, These two sampling areas were selected due to their differences in agroecological zone (representing mid-land and highland) and proximity to sample processing laboratories, to see abundance of EPF across two

agroecological zones. Specifically, soil samples were collected from the grazing area, lake side and forest areas of tow agroecological zones. During collection, geographical coordinates (X, Y) and elevations of each sampling points were recorded using a Geographic Positioning System (GPS) mobile applications. From each sampling points, approximately 1kg of soil samples were collected from the surface to a depth of 15 cm using a shovel (Gebremariam *et al., 2021*).

To avoid cross-contamination, the shovel was sterilized by immersing it in 70% ethanol between sampling points. These soil samples were collected in a sealed sterile polythene bag, labeled, properly stored in the ice box and were transported to laboratories. Among the eighty (80) soil samples collected, forty (40) samples were taken to the Microbiology laboratory of the College of Veterinary Medicine and Agriculture (CVMA) based in Bishoftu for isolation of fungi using artificial media and the rest forty (40) samples were taken to Ambo Agricultural Research Center Laboratory to process and isolate EPF using insect baits. Before proceeding to isolate EPF using artificial media, the soil samples were shade-dried sieved to remove any residues, grounded into a fine powder, sieved again, and preserved for further use as previously described (Mesquita *et al.,* 2022).

3. 4.2. Preparing artificial selective media and Fungi isolation

The artificial selective medium was prepared from 65 grams of Sabouraud dextrose agar (SDAY) and 2.5 grams of yeast aseptically weighed on a balance and poured into an Erlenmeyer flask (Appendix 4A). Then, one liter of distilled water was added, mixed well, dissolved by heating, stirred frequently, and boiled for one minute until completely mixed. It was covered with aluminum foil and sterilized in an autoclave at 121°C for 15 minutes at a pressure of 15 pounds (irritable bowel syndrome). It was then allowed to cool to a temperature of 40 °C in a water bath, and 100 µg/ml chloramphenicol was added to the medium to be used as an antibiotic against bacteria growth on the media, and then poured into the sterilized plates and incubated at 25±1°c for 24 hours to check for sterility. After sterility was confirmed, it was allowed to be used for EPF identification (Correa *et al.,* 2022).

One gram of sieved soil sample was mixed with 9ml (1:9) of sterilized distilled water in a test tube and vortexed for 10 min to produce a homogenized dilution. Then a serial dilution(up to 10^{-3}) steps were made to homogenize the suspension and then 100 µL of the

solution was removed and poured into Petri dishes containing SDAY medium The suspension was evenly distributed on the surface of the medium using sterilized spatula and then incubated at 25 ± 1°C and a relative humidity of ≥ 80% for approximately 21 days until spores formed. Three replicates of the dilution were performed for each isolates. Single colony subcultures were repeatedly done on the Potato dextrose agar (PDA) media until a pure colony was formed (Gürlek *et al*., 2018).

3. 4. 3. Rearing of Galleria Mellonella for Fungi Isolation

Entomophatogenic fungi were also isolated from the sampled soil using the Galleria bait method as described by Meyerling and Eilenberg (2006). Adult *Galleria mellonella* (*G. mellonella*) used as insect bait for EPF isolation was obtained from Ambo Agricultural Research Center. The rearing of *G. mellonella* was carried out in plastic boxes that were incubated in a dark incubator at 20 °C according to Meyling in 2007. Adult moths (1:1 female to male ratio) were maintained in a bottle jar by providing honey and water (Appendix 5). Additionally, folded tissue paper was placed in bottle jar for adult moths to lay eggs. The paper was removed with the eggs attached and placed in a new plastic container with food ingredients which comprises 180 g honey, 180 g glycerin and 50 g wheat bran.

For hatched larvae, honey and glycerin were melted in a saucepan and after 15 minutes they were thoroughly mixed with wheat bran (Appendix 5). The tissue papers attached to food and eggs were transferred to a larger box for rearing and incubated at 20 °C. The food was changed regularly according to the larval growth stage of *G. mellonella*. The resulting fourth to fifth instar larvae (approximately 30 days after hatching) were used as bait (Keno *et al.,* 2022) (Appedix 5C).

3. 4. 4. Isolation of Entomopathogenic Fungi with G. mellonella bait

Entonopathogenic fungi were isolated from the soil samples using *Galleria mellonella* bait as per the methods and protocols described previously (Meyerling and Eilenberg, 2006). *Galleria mellonella* larvae with a length of approximately 2.5–3 cm (4 weeks after hatching) were used to bait EPF in the soil samples (Meyerling, 2007). Before inoculation in the soil, the larvae were immersed in boiling water (at 56 °C) for 10 seconds and transferred to running bath water for 30 seconds to cool and prevent the formation of

excessive webbing in the soil. About 1 kg of each soil sample was moisturized with sterile water and then filled into a glass container with a screw cap, leaving some space on the top to inoculate *Galleria* larvae. Ten (10) *G. mellonella* larvae were inoculated into each glass container and incubated at 25±2 °C in the dark. To ensure permanent soil contact, all containers were inverted every other day.

Dead larvae were removed every three days from the glass containers for ten days. The moisture level was maintained by moistening with sterile water each time, following the inspection of the dead larvae. The dead larvae were collected and immersed in 1% sodium hypochlorate and 70% ethanol for one minute each and washed in sterile distilled water for three minutes to remove saprophytes organisms and all conidia that were on the outer surface of the larval body. The disinfected cadavers (dead larvae) were allowed to dry on filter paper for three minutes (Meyerling and Eilenberg, 2006). This step was necessary to ensure that the mycosis observed on the surface of the insect cadaver was not due to the spores on outer surface, but rather to show the growth from the inside of the insect outward after colonization of internal organs. A larva was considered to have mycosis if the growth of the fungus was visible on the external surface and those showing hyphal growth characteristics of the EPF were recorded as infected and the EPF were isolated in triplicate according to the guidelines and methods previously described (Mekonnen, *et al.,* 2024).

Observation of spores of fungi on the cadavers *of G. mellonella* larvae was followed by transfer of fungi to SDA medium prepared using the same techniques as for artificial selective media. Then the inoculation medium was sealed with paraffin film and left in the dark at 25 ± 1 °C and a relative humidity of ≥ 80% for approximately 21 days until spores formed. Subcultures of each colony were performed several times until a pure colony was obtained for the experiment.

3.4. 5. Microscopic examination and identification of Fungi

Fungal elements were stained using Lactophenol cotton blue and were examined under a microscope for morphological identification as previously described (Gebremariam *et al., 2021*). Lactophenol cotton blue comprises phenol (kills the organisms), lactic acid (preserves the fungal structures), and cotton blue (stains the chitin in the fungal cell walls). After staining with Lactophenol cotton blue, entomopathogenic fungi were identified to the species level based on their microscopic morphology. To perform lactophenol staining,

four-stage activities were performed including a drop of lactophenol-cotton blue solution was placed on a clean microscope slide, then a small amount of fungal culture (mycelial mat) was then removed from the edge to have young colonies using a sterilized needle and a fungal culture was prepared on the slide. The second needle was used to pull out the fungal structures, and then the coverslip was carefully placed on the slide by pressing down and avoiding air bubbles and finally entomopathogenic fungal species were identified (Gebremariam *et al., 2021*).

Next, the genus Beauveria was identified under the high objective power of the compound microscope based on the morphological characteristics and taxonomic features of the conidia such as conidiogenic cells: densely bundled or in whorls or solitary, colorless, short, with a spherical or flask-shaped base extending apically and at a short distance below each of several apically shaped cells repeatedly branched conidia (Imoulan *et al.,* 2016) (Figure 4A). Similarly, the genus Metarhizium was identified using taxonomic characteristics including conidiogenic cells with rounded to conical tips are arranged in dense hymenium; Conidia aseptate, cylindrical or ovoid, form chains that aggregate into prismatic or cylindrical columns or a solid mass of parallel chains, ranging from pale to light green to yellow-green, olive green, sepia or white mass (Imoulan *et al.,* 2016) (Figure 4B). Finally, the taxonomically characterized fungi were identified at both genus and species levels based on their morphological characteristics and microscopic analyzes (Imoulan *et al.,* 2016).

3. 4. 6. Prescreening of Fungal isolates viability by germination mean percentage

Prior to the preparation of the working concentration, the viability of the isolates of the EPF was confirmed through conidial spore germination test using the previously described standard procedures (Ayele *et al.,* 2020). For this purpose, fungal spores were collected from the 3-week-old culture by scraping with a sterilized spatula. Then the collected spores were added into 10 ml of sterile distilled water supplemented with Tween 80 (0.1% v/v) as a surfactant in falcon tube and evenly mixed through vortexing at 300rpm (Msagni, 2022). Spore concentration was adjusted as 1 x 10^6conidia/ml by using improved Neubauer hemocytometer under light microscope. Then following this 100-µl suspension was spread over the fresh PDA and 2 sterilized glass slides were laid over inoculated medium and incubated at 25 °C for 24 hrs. Then after 24 hrs of incubation, over germination progresses of spores were halted by ethanol (70%) dispensing. Then 100 spores of both germinated

and non-germinated spores were counted on microscopic glass slides under the x 40 magnifying objectives of light microscope and the experiment was repeated three times. Germinated spores were characterized with germ tubes of long spores appearance.

On observation in the present experiment, all isolates showed a good mean germination percentage. However, it was difficult to use the whole (17) fungal isolate . Hence, Based on the previously described method by Gebremariam *et al. (2021),* seven isolates with germination mean of >95% were considered the best and selected for the experiment

3. 4.7. Preparation of Entomopathogenic fungi concentration

The stock suspension was prepared when the gently scraped spores from the medium were suspended in 10 ml of sterile distilled water containing 0.01% Tween80 (Appendix 6). Then the fungal suspensions were vortexed for 10 minute to break up the conidial chains or clumps and filtered through several layers of cheesecloth to remove mycelia. The conidial dose in the filtrate was estimated to be 1.1 x 10^9 conidia/ml using a hemocytometer under a light microscope (40x magnification) and further diluted to obtain the working concentrations of 1.0 x 10^6 conidia/ml and 1.0 x 10^7 conidia/ml and 1.0 x 10^8 conidia/ml by adding sterile distilled water containing 0.01% Tween80 (Murigu *et al.,* 2016).

3. 5. Tick Collection, Transportation and Identification

Tick infested cattle were purposefully selected to collect tick species from their bodies. First, the animal was appropriately restrained to avoid possible injuries to itself and sample collectors. During the collection process ticks were carefully removed from the animal body by bending up and forward and then pulled perpendicularly. To prevent sepsis of the collation body sites of cattle disinfectants such as alcohol and gloves were used. The collected ticks were then placed in ventilated tubes with absorbent materials such as cotton and were transported to the parasitology laboratory of the Addis Ababa University College of Veterinary Medicine and Agriculture. In the laboratory ticks were identified to species level based on morphological characteristics such as mouthparts, coxa I, patterns of enamel ornamentation, leg color, festoons and ventral body parts (Walker *et al.,* 2003). *Amblyomma variegatum,* the most common and predominant tick species and economically one of the most important species in the study area was selected for the

present experiment and hence an overall of 1050 ticks were used for the in vitro efficacy of seven (7) selected fungi isolates in the present study.

3. 6. Invitro Efficacy of Entomopathogenic Fungi against Adult *A. Variegatum*

TicksThe in vitro efficacy of EPF was tested using the adult immersion test (AIT) on adult *A. variegatum* ticks which was performed as previously described by De Sousa et al. (2020).In brief, ten (10) active live adult A. variegatum ticks were added to the Petri dish in three replicates of each concentration, and then 3 mL of each concentration was added directly to the three replicate of Petri dishes. After immersion for 5 minutes, then the ticks were placed in other sterilized petri dishes containing 11cm Whatman filter paper to dry the ticks and incubated at 28 °C and 80% relative humidity for 15 days.

Each tick in each Petri dish was closely examined under a stereomicroscope for any mortality data every two days for fifteen (15) days, starting from day three post-inoculation. Meanwhile, Amitraz 12.5% (Acarmic, India), currently on market in Ethiopia and used by the community, was used as a positive control as per the manufacturer's recommendations, while three milliliters of distilled water were used as a negative control. The pathogenicity of the fungi was suggested to be confirmed when it killed the tick and grew on their cadavers. The percentage mortality was calculated using a formula given by Kemal *et al.,* 2020, as follows:

Mortality rate = (Number of dead ticks ÷Total number of ticks) × 100.

3. 7. Data Analysis

The data collected was stored in a Microsoft Excel dataset framework and checked for missing values and errors. Descriptive statistics were used to summarize the data into means, percentages, and standard error. Data analysis was peformed using STATA version 14. One-way analysis of variance (ANOVA) was used to analyze tick mortality and t-test was used to compare the mean avaialability of fungal in two different Agroecological Zones. While the paired wise mean comparison test (Tukey test) was used to test mean difference between dose concentrations level at a significance level of 5%. Probit regression analysis was used to analyze the lethal concentrations (LC_{50} and LC_{99}) for tick mortality data. In all analyses, the confidence level was maintained at 95% and the p value was assumed to be less than 5%.

3. 8. Ethical Clearance

The study received ethical approval for the research from the College of Veterinary Medicine and Agriculture (CVMA) at Addis Ababa University, per minutes of the animal research ethical review committee (reference number VM/ERC/02/23/16/2024, and was issued certificate reference number VM/ERC/02/16/024, 26/03/2024.

4. RESUTS

4. 1. Demographic Characterictics and Livestock Manegment of the Respondents

The interview involved a total of one hundred and eighty (180) participants, including 128 men and 52 women. The age of the respondents was from 18 (minimum) to 73 (maximum) years with standard deviation=14.50 (Table 1). The majority of animal owners (80.5%) maintained mixed livestock species in their homes Moreover, the most commonly kept breed of cattle were zebu, with 74.4% of owners raising this breed, followed by mixed breeds (18.33%) and exotic breeds (7.2%).

The majority of the interviewed, 141 (78.3%), keep their animals for draft and milk production purposes, the others, 25 (13.9%), keep them for draft purposes and 10 (5.5%) for milk production and 4 (2.2%) for fattening only (Table 1). Most livestock owners, 157 (87.2%), use extensive management systems, while 17 (9.4%) use semi-intensive management systems and 6 (3.3%) use intensive management systems

Table 1: Socio demographic characteristics of the respondents

Variables	Category	N	Percent	mean	95%CI
Gender	Male	128	71.11		
	Female	52	28.89	1.71	1.64-1.78
	18-25	24	13.00		
Age	26-30	34	18.89		
	31-40	32	17.78	3.47	3.24-3.70
	41-50	30	16.67		
	51-60	43	23.89		
	>60	17	9.44		
	Diploma	9	5.00		
	Degree	14	7.78		
Education	Elementary	52	28.89	4.03	3.82-4.24
	High School	23	12.78		
	Illiterate	51	28.33		
	Religious school	31	17.22		
Livestock species	Cattle	34	18.89	1.82	1.76-1.88
	Mixed	145	80.56		
	sheep	1	0.56		
Cattle breed	Zebu	134	74.44		
	Exotic	13	7.22	2.56	2.45-2.68
	Both	33	18.33		
	Draught	25	13.89	3.48	3.33-3.64
Purpose	draught and milk	141	78.33		
	Fattening	4	2.22		
	Milk	10	5.56		
	Extensive	157	87.22	1.22	1.13-1.31
Management	Semi-intensive	17	9.44		
system	Intensive	6	3.33		

4. 2. Knowledge of the owners on tick control

Based on their KAP assessment, all 180 (100%) respondents had awareness about ticks and their impacts (Fig.2) and the majority (91.7%) respondents gave first rank for tick in terms of negative impacts. Regarding the livestock species more affected by ticks, 168 (93.3%) of respondents indicated that cattle are the most affected as compared to other livestock species whereas most of the respondents (72.2%) indicated that they know the names of some tick species in their own language that affect different species of animals (Fig.2). Less than half percent (44.4%) of livestock keepers believed that exotic breeds of animals are more susceptible to tick infestation. Majority of them (64.4%) said that tick infestation is more common at the beginning of the rainy season.

Regarding cattle age groups, 43(23.9%) of respondents replied older cattle are more affected than adult and youngers, whereas regarding animal sex category, 29 (16.1%) owners indicated that both sexes are equally affected (Fig 2). The majority (88.3%) of the respondents said the color of the animals was not a factor in tick infestation in their animals (Fig 2).The interviewee was also questioned regarding the negative effects of tick infestations on their animals. Thus, 91 respondents (50.6%) know that tick infestation results in weight loss in the animals, damages the teats of female animals and a loss of production.

Less than half (40.6%) of interviewed respondents know that ticks bite humans, while the majority of them believed that ticks do not bite humans or they do not know whether ticks bite humans or not. In addition to this, only 16.7% of respondents know that tick bite can transmit diseases to humans, and the rest of the respondents replied they do not know whether ticks transmit or not. Majority of the respondents (94.4%) replied that the source of ticks to infest their animals is the grazing areas and grass covered areas and majority (72.2%) of the owners know about the negative impacts of acaricides on human health and the environment (Fig 2).

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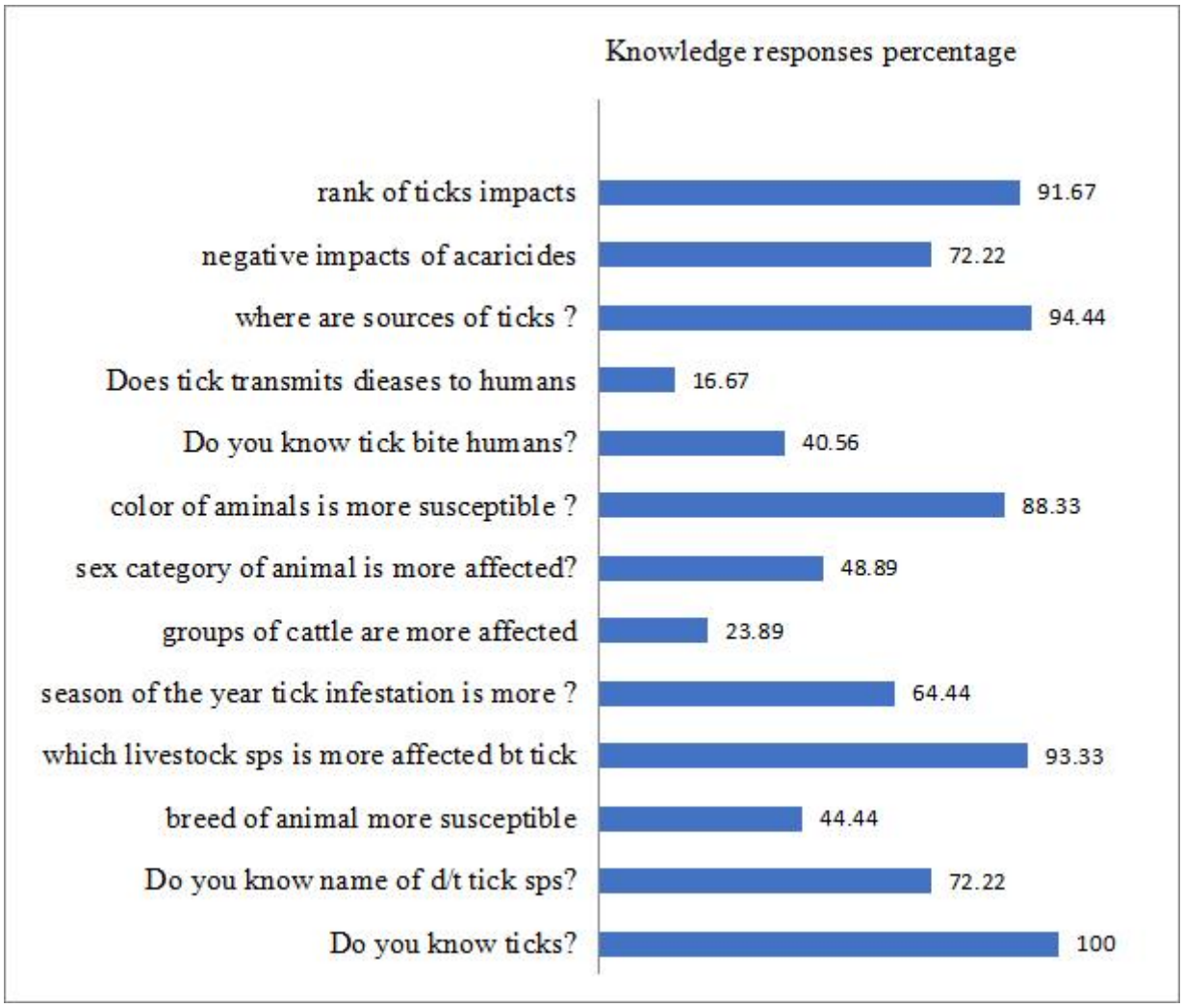


Figure 2: Knowledge positive responses of livestock keepers for to words tick control

4. 3. Attitude of the owners towards tick control

Regarding the chemical acaricidal application, only a small number (4.4%) of the owners said that both the owners and professionals like veterinarians and CAHWs are responsible for applying the chemicals to control tick infestations (Fig.3). The majority of owners (91.7%) believed that they noticed tick resistance against and to overcome the problem; 121 (67.2%) believed in the changing of existing acaricides and replacing them with other alternatives.

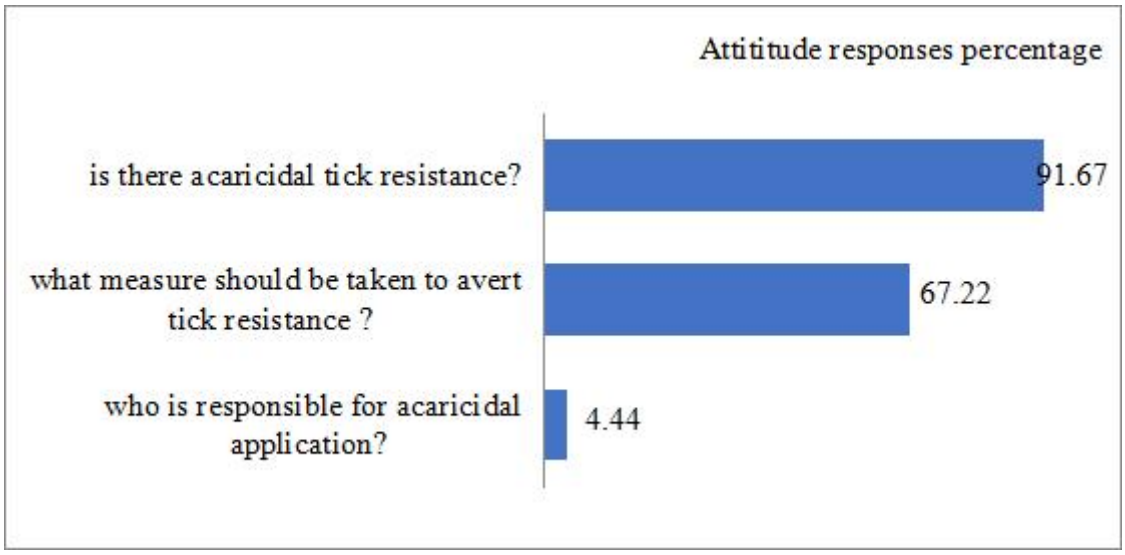


Figure 3: Attitude positive responses of livestock keepers to control tick infestation.

4. 4. Practices of the owners regarding control of tick infestation

Results of the present questionnaire survey indicated that majority of the respondents (91.7%) used injection forms of acaricides to control ticks and ivermectine is the most frequently used by majory (88.9%) of owners and high number the respondents (93.9%) preferred this acaricide duet its effectiveness and safety compared to the rests such as Amitraz and Diaznon. An overall of 112 (99.44%) reported that the source of acaricdes was from legal private drug stores and government veterinary clinics (Fig 4).

All the respondents (100%) did not get any training methods of application of the acaricidal drugs. They also added that they are applying the chemicals by them theselves without any protective equipment. Majority of (99.44%) the respondents informed that they use the spraying methods to apply acarididal chemicals to their animals aand near to all (99.44) of the respondents use the hand sprayer equipment to apply (Fig.4).

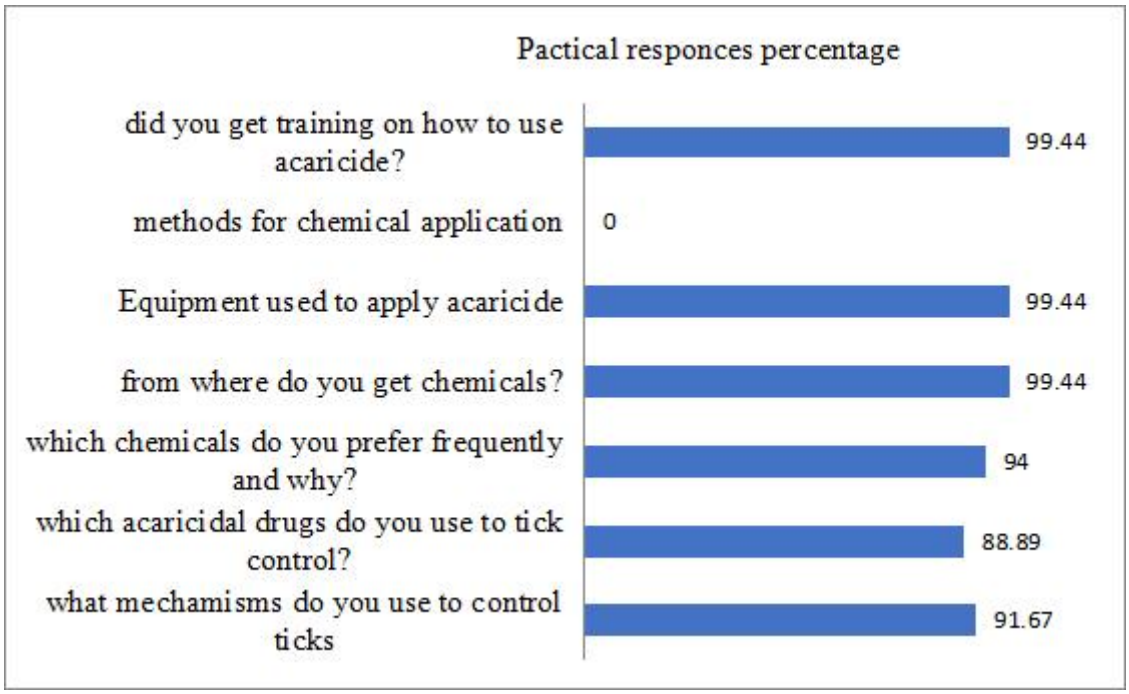


Figure 4: Acaricide drugs and equipment usage practicces to control ticks

4. 5. Association of KAP with demographic characteristics

Knowledge score assessment of the present study revealed that 47.8% of respondents have good knowledge about the impact of tick infestation on the economy and the negative impact of acaricides on the environment and human health, but 52.2% of them have poor knowledge regarding the negative impact of acaricides (Table 2). The majority (83.9%) of respondents have good practices of controlling tick infestations, while 16.1% of respondents lack this good practices. An overall of 70.6% of respondents have a positive attitude towards the preference of the person using acaricides on their animals and the finding for alternative acaricides, while 29.4% of respondents showed a negative attitude and believed that there is no tick resistance to the current acaricides exist (Table 2).

Table 2: Proportion of KAPscore towards ticks and tick control

KAP	Number of respondents	Number of questions	Mean score	Range score	High	Low
Knowledge	180	13	6.5	3−10	47.8%	52.2%
Attitude	180	3	0.8	0−2	70.6%	29.4%
Practice	180	7	5.7	2−7	83.9%	16.1%

Logistic regression analysis showed that respondents with the age of greater than 60 years were 9.35 times more likely (95% CI = 1.60−54.76) to have sufficient knowledge about the health and economic impacts of tick infestation and negative effects of acaricides in the environment and on human health than those respondets with the age range of 18 to 25year-old age group (Table 3).

Table 3: Association of knowledge score with socio-demographic variables.

Variables	Category	N	Knowledge score		Logistic regression		
			Adequate	Inadequate	OR	95%CI	P-value
Gender	Male	128	69(53.9%)	59(46.1%)	2.04	0.98− 4.24	0.057
	Female	52	17(32.7%)	35(67.3%)	Ref.		
Age category	18-25	24	9(37.5%)	15(62.5%)	Ref.		
	26-30	34	13(38.3%)	21(61.77%)	1.34	0.414−.36	0.626
	31-40	32	13(40.6%)	19(59.4%)	1.54	0.465−.20	0.484
	41-50	30	15(50%)	15(50%)	2.36	0.658−.55	0.192
	51-60	43	22(51.2%)	21(48.84%)	2.16	0.578−.12	0.256
	>60	17	14(82.35%)	3(17.65%)	9.35	1.605−4.76	0.013**
Education	College	9	4(4.65%)	5(5.32%)	Ref.		
	Degree	14	6(42.86%)	8(57.14%)	1.25	0.217−.30	0.805
	Elementary	52	18(34.6%)	34(65.4%)	.77	0.17−3.54	0.736
	High School	23	13(56.52%)	10(43.48%)	1.99	0.40−9.85	0.395
	Illiterate	51	31(60.8%)	20(39.2%)		0.27−0.37	0.765
	Religious school	31	14(45.16%)	17(54.84%)	1.28	0.14−3.82	0.118
Total		180	86(47.8%)	94(52.2%)			

N=number of respondents, *Ref=Reference of correlation;* OR=Odds ratio, CI=Confidence interval, **=statistically significant**.**

Similarly, the logistic regression analysis revealed the presence of statistically significant difference ($P<0.05$) in attitude between genders. Consequently, male respondents were 2.32 times more likely (95% CI = 1.10–4.89) to have a positive attitude toward tick infestation control than female respondents (Table 4).

Table 4: Association of attitude score with socio-demographic variables

Variables	Category	N	Attitude score		Logistic regression		
			Positive	Negative	OR	95%CI	P-value
Gender	Male	128	97(75.78%)	31(24.22%)	2.32	1.10−4.89	0.027**
	Female	52	30(57.70%)	22(42.30%)	Ref		
Age category	18-25	24	18(75%)	6(25%)	Ref		
	26-30	34	27(79.41%)	7(20.59%)	1.47	0.38−5.65	0.575
	31-40	32	21(65.63%)	11(34.37%)	1.02	0.27−3.86	0.972
	41-50	30	21(70%)	9(30%)	1.20	0.30−4.90	0.809
	51-60	43	29(67.44%)	14(32.56%)	1.11	0.26−4.81	0.890
	>60	17	11(64.70%)	6(35.30%)	1.12	0.20−6.14	0.896
Education	College	9	7(77.78%)	2(22.22%)	Ref		
	Degree	14	11(78.57%)	3(21.43%)	1.38	0.17−11.32	0.766
	Elementary	52	38(73.0%)	14(27%)	1.04	0.17−6.15	0.968
	High School	23	19(82.60%)	4(17.40%)	1.54	0.22−10.58	0.663
	Illiterate	51	30(58.82%)	21(41.18%)	0.51	0.08−3.23	0.474
	Religious school	31	22(70.97%)	9(29.3%)	0.88	0.13−6.11	0.899
Total		180	127(70.6%)	53(29.4%)			

N=number of respondents, *Ref=Reference of correlation;* OR=Odds ratio, CI=Confidence interval, **=statistically significant**.**

Respondents whose ages were 31–40, 51–60, and above 60 years were 0.10 times (OR 95% CI = 0.01−0.97), 0.07 times (95% CI = 0.01−0.74), and 0.03 times (OR 95% CI = 0.00-0.42) less likely to have good practices of control of tick infestation than those respondents with 18–25 age groups, respectively (Table 5).

Table 5: Association of practices score with socio-demographic variables

Variables	Category	N	Practical score		Logistic regression		
			Good	Poor	OR	95%CI	P-value
Gender	Male	128	106(82.81%)	22(17.19%)	0.97	0.352−0.70	0.951
	Female	52	45(86.54%)	7(13.46%)	Ref		
Age category	18-25	24	23(95.84%)	1(4.16%)	Ref		
	26-30	34	33(97.06%)	1(2.94%)	1.07	0.061−9.00	0.962
	31-40	32	25(78.12%)	7(21.88%)	0.10	0.01−0.97	0.047**
	41-50	30	25(83.30%)	5(16.70%)	0.10	0.011−0.14	0.064
	51-60	43	34(79.70%)	9(20.93%)	0.07	0.010−0.74	0.027**
	>60	17	11(64.70%)	6(35.30%)	0.03	0.000−0 .42	0.009**
Education	College	9	8(88.89%)	1(11.11%)	Ref		
	Degree	14	12(85.71%)	2(14.29%)	0.64	0.041−0.33	0.750
	Elementary	52	46(88.46%)	6(11.54%)	1.07	0.091−1.99	0.955
	High School	23	19(82.61%)	4(17.39%)	0.38	0.034−0.84	0.454
	Illiterate	51	39(76.47%)	12(23.53%)	1.30	0.121−4.20	0.829
	Religious school	31	27(87.10%)	4(12.90%)	2.42	0.203−0.84	0.495
Total		180	151(83.9%)	29(16.1%)			

N=number of respondents, *Ref=Reference of correlation;* OR=Odds ratio, CI=Confidence interval, **=statistically significant**.**

4. 6. Isolation of Entomopathogenic fungi

In the present study an overall of 80 soil samples including 35 from forests, 25 from grazing areas, and 20 from lake sides were collected, out of which a total of 17 (21.2%) soil samples yielded entomopathogenic fungi isolates which belonged to the genera of *Beauveria* and *Metarhizium* isolates using artificial selective media and *Galleria mellonella* as insect bait methods. The prevalence of genus *Metarhizium* 12.5% (10/80) was higher than the genus *Beauveria* 8.75% (7/80). The soil samples obtained from Burayu agro-ecology showed a higher fungal prevalence (Table 6) than that of Bishoftu agro-

ecology, and soil samples from forest habitates were with higher prevalence than those samples from the grazing areas (Table 6).

Table 6: Entomophatogenic fungi isolates with their location and habitats

S/N	Species	Isolates	Location	Habitat	Altitude	Latitude	Longitude	Soil type
1	Metarizhium	FS2	Bishoftu	forest	2114	85.157	39.127	vertisol
2	Beauveria	FS7	Bishoftu	forest	2003	85.133	39.125	vertisol
3	Beauveria	FH11	Bishoftu	forest	1818	84.61	38.5937	vertisol
4	Metarizhium	FS15lk	Bishoftu	Lake	1854.8	84.534	38.595	vertisol
5	Beauveria	BSF1	Burayu	grazing	2640	94.33	38.3653	loam
6	Metarizhium	BSF2	Burayu	forest	2633	94.33	38.3652	loam
7	Metarhium	BSF4m	Burayu	forest	2633	94.34	38.3652	loam
8	beauveria	BSF4	Burayu	forest	2633	94.34	38.3652	loam
9	Metarizhium	BSF6	Burayu	forest	2641	94.34	38.653	loam
10	Metarizhium	BSF10	Burayu	grazing	2633	94.16	38.3631	loam
11	Metarizhium	BSF12	Burayu	forest	2632	94.17	38.3634	loam
12	Metarhizium	BSF13m	Burayu	forest	2635	94.16	38.3634	loam
13	Beauveria	BSF13	Burayu	forest	2635	94.16	38.3634	loam
14	Metarizhium	BSF14	Burayu	forest	2635	94.17	38.3643	loam
15	Beauveria	BSF15	Burayu	forest	2634	94.17	38.3637	loam
16	Beauveria	BSF20	Burayu	forest	26131	94.38	38.36548	Loam
17	Metarhizium	BSF37	Burayu	forest	2648	94.39	38.3646	Loam

4. 7. Identification of Entomopathogenic fungi

On observation, *Metarhizium anisopliae* showed *Colonies* characteristics such as initially white or creamy mycelium, becoming shades of green or yellow to shades of dark green during sporulation (Table7). Conidiophores were simple or double-branched with an elongated shape (Fig. 5 A, B).

Table 7: Morphological and cultural characteristics of *Metarhizium anisopliae* isolates

No	Isolates	Colony color		Colony shaped	Colony texture	Elevation	Shapes of spores
		Front side	Reverse side				
1	FS2	light green	orange	round	powder	flat	cylindrical
2	FS15lak	dark green	brown	round	powder	flat	ellipsoidal
3	BSF2	dark green	orange	round	powder	flat	cylindrical
4	BSF4m	light green	orange	round	powder	flat	cylindrical
5	BSF6	light green	orange	round	powder	flat	ellipsoidal
6	BSF10	light green	orange	round	powder	flat	cylindrical
7	BSF12	light green	brown	round	powder	flat	cylindrical
8	BSF13m	dark green	orange	round	powder	flat	cylindrical
9	BSF14	dark green	brown	round	powder	flat	ellipsoidal
10	BASF37	light green	brown	round	powder	flat	ellipsoidal

The surface of the colony of *Beauveria bassiana* was white to cream and fluffy to powdery (Table 8). It was usually white becoming slightly yellowish over time (Fig. 5 C).The fungal spore shape was globular (fig 5 D).

Table 8: Morphological and cultural characteristics of *Beauveria bissiana* isolates

No	Isolates	Colony color		Colony shaped	Colony texture	Elevation raised	Shapesof spores
		Front side	Reverse side				
1	FS7	White	White	round	powder	raised	globose
2	BSF1	White	White	round	powder	raised	globose
3	BSF4	White	White	round	powder	raised	globse
4	FH11	White	White	round	powder	raised	globose
5	BSF13	White	White	round	powder	raised	globes
6	BSF15	White	White	round	powder	raised	globose
7	BSF20	White	White	round	powder	raised	globose

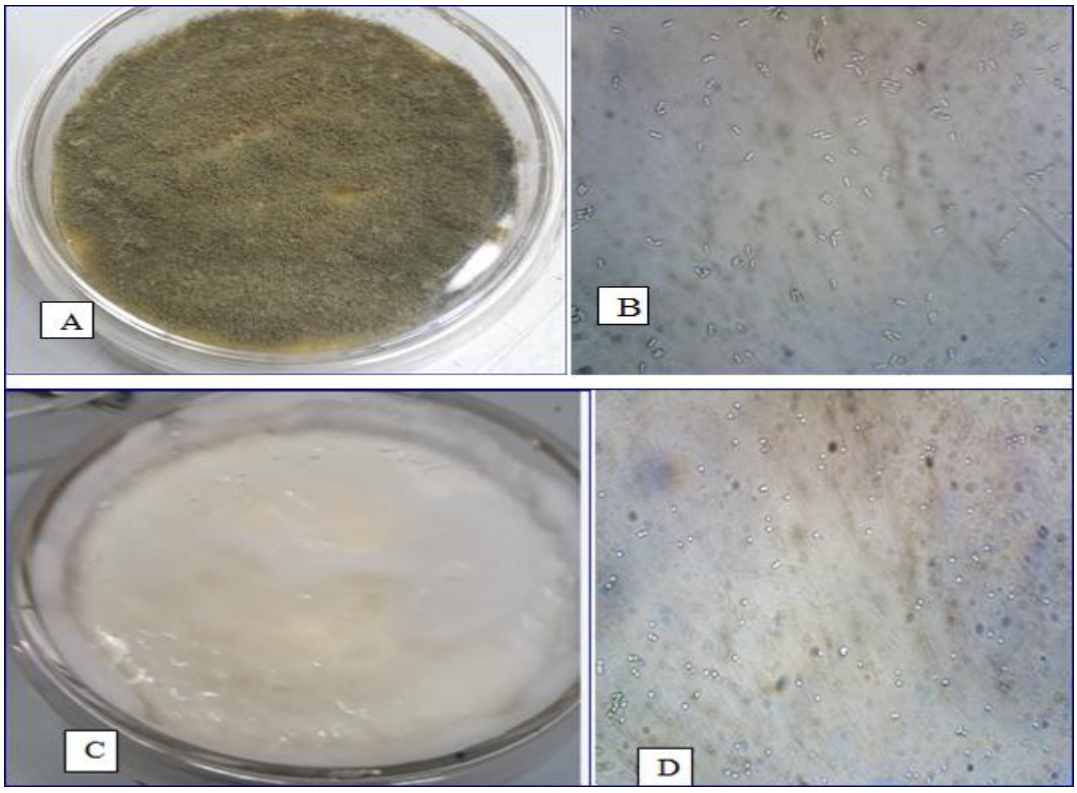


Figure 5: Identification of the two EPF genera

Photos of *Metarhizium anisoplia* colony (A) and its rode shaped spores (B and *Beauveria bissiana* colony(C) and its Spherical shaped spores (D) under microscope.

Regarding the availability, 80% (8/10) Metarhizhium and Beauveria 71.43% (5/7) were from Burayu Agroecological zone, whereas 20%(2/10) and 28.57% (2/7) Metarhizium and Beveauveria respectvelly from Bishoftu Agroecological zone (Fig 6).

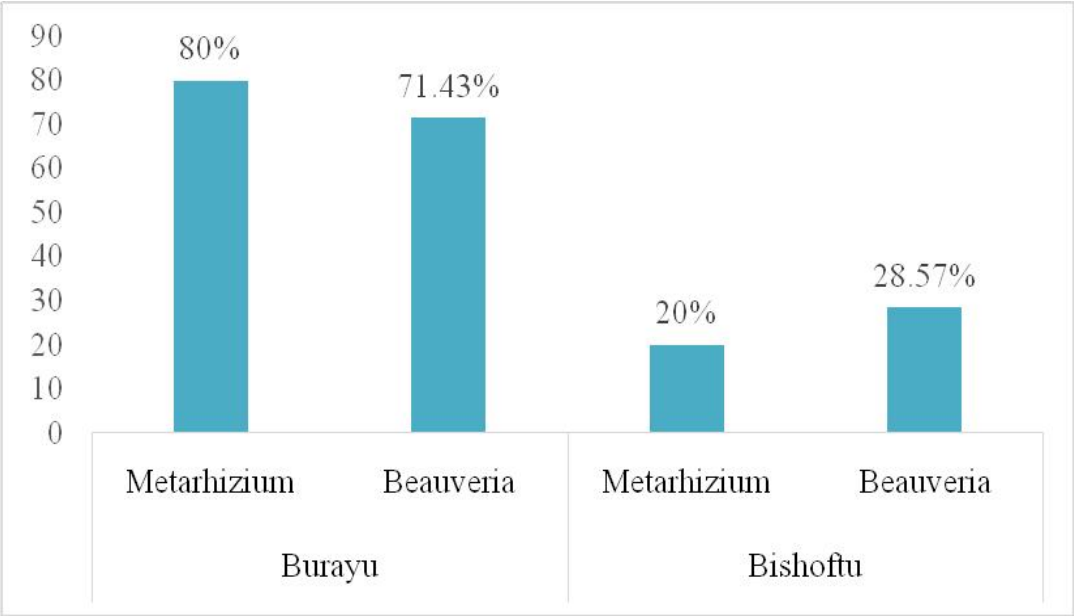


Figure 6: Prevalence of EPF isolated from Burayu and Bishoftu soil samples

For each isolate, the mean percentage of germination was calculated and ranged from 88% (minimum) to 98% (maximum) (Table 9).

Table 9: Mean percentages of germination of EPF

Isolates	Genus	Germination mean(%)±SE	%95 CI
FS1	Metarhizium	98±0.01	95.00−100.00
FS7	Beauveria	97±0.02	94.00−100.00
FH11	Beauveria	92±0.02	83.84−5.67
FS15lak	Metarhium	89±0.31	82.769−5.00
BSF1	Beauveria	90±0.03	84.029−6.00
BSF2	Metarhizium	95±0.01	92.01−9.47
BSF4m	Metarhizium	98±0.01	95.21−00.79
BSF4	Beauveria	97±0.02	93.60−00.40
BSF6	Metarhizium	87±0.02	83.61−0.40
BSF10	Metarhizium	90±0.03	84.03−5.99
BSF12	Metarhizium	88±0.03	81.52−94.48
BSF13m	Metarhizium	94±0.02	90.01−7.90
BSF13	Beauveria	89±0.03	82.76−95.24
BSF14	Beauveria	89±0.03	82.76−95.24
BSF15	Metarhizium	98±0.01	95.21−00.79
BSF20	Beauveria	96±0.02	92.11−9.91
BSF37	Metarhizium	95±0.02	90.66−9.35

Out of the 17 isolates of EPF identified only those with germination mean percentages ≥ 95% were selected for the current in vitro efficacy experiment (Table 10).

Table 10: Isolates of EPF selected for the in vitro efficacy experiment

Isolates	Genus	Germination means(%)±SE	%95CI
FS7	Beauveria	97±0.02	94.00−100.00
BSF2	Metarhizium	95±0.01	92.00−99.47
BSF4m	Metarhizium	98±0.01	95.21−100.79
BSF4	Beauveria	97±0.02	93.60−100.40
BSF15	Metarhizium	98±0.01	95.21−100.79
BSF20	Bauveria	96±0.02	92.10−99.91
BSF37	Metarhizium	95±0.02	90.65−99.35

4. 8. Effectiveness of Artificial Media and *Galleria mellonella* bait Methods

Galleria bait method appeared to be the better method with fourteen (14) positive results while artificial selective media was able to yield only three (3) positive isolates. Hence statistically significantly (p=0.0023) higher mean was recorded using t-test in *Galleria bait* methods (Table11).

Table 11: Mean differences in between of artificial media and *G. mellonella* methods

Variable	Obs.	Mean±SE	Std.Dev	95% CI	t-test	p-value
G.bait method	40	0.35±.076	0.48	0.196−0.505		
					3.15	0.0023
S.Artificial media	40	0.075±0.04	0.27	0.010−0.160		
Total	80	0.2125±.04	0.41	0.121−0 .304		
difference		0.275±0.09		0.101−0.449		

G=Galleria Obs= Observation, S=Selected SE= Standerd Error, Std.Dev= Standerd deviation, CI= Confidence Interval.

4. 9. Invitro Efficacy of Entomopathogenic Fungi against *A.Variegatum*

Ticks Based on the in vitro efficacy of three different concentration levels (1x106, 1x107and 1x108 conidia/ml) recorded for 15 days post exposure (Table 12) ,there was significance different in between fungal isolation BSF4m and Amitraz (positive control) in mortality of A. variegatum at $P<0.05$ (Table 12).

Table 12: The mean death of *A. variegatum* ticks recorded for 15 days

No	Isolates	Day3	Day5	Day7	Day9	Day11	Day13	Day15
1	FS7	47±5.76	59± 6.32	72±6.39	77±5.09	81± 5.2	89±5.24	90±4.32ab
2	BSF2	50± 5.76	58 ± 6.32	64±6.39	74 ±5.09	79± 5.2	89±5.24	91±4.32ab
3	BSF4m	61± 5.76	65± 6.32	72±6.39	81±5.09	86±5.2	96±5.24	98±4.32b
4	BSF4	57± 5.76	64± 6.32	66±6.39	77±5.09	81±5.2	89±5.24	97±4.32ab
5	BSF15	59± 5.76	63± 6.32	68±6.39	75±5.09	81±5.2	91±5.24	96±4.32ab
6	BSF20	65± 5.76	67± 6.32	71±6.39	83±5.09	93±5.2	95±5.24	94±4.32ab
7	BSF37	57± 5.76	63± 6.32	72±6.39	75±5.09	80±5.2	89±5.24	94±4.32ab
8	PC	65±3.77	67± 4.14	68±4.18	71±3.33	73±3.40	75±3.43	76±2.83a
9	NC	00±3.77	00±4.137	00±4.18	00±3.33	00±3.40	00±3.43	00±2.83c

Note: Means sharing a letter in the group label is not significantly different at the 5% level. PC=Positive control, NC=negative control.

Although a statistically significant difference was not recorded in between the two genera, Metarhizium showed a more effect than the genus Beauveria against adult *A.vareigatum* ticks (Table 13).

Table 13: In vitro efficacy of EPF against *A. variegatum* ticks

Fungal genera	concentration	tick treated	%Mean death ±SE	95% CL
Metarhizium	$1X10^6$	120	83.25±2.30	78.34 −88.16ab
	$1X10^7$	120	93.00 ±2.30	88.09−97.90bc
	$1X10^8$	120	97.50± 2.30	92.59−102.40c
	PC	120	74.00± 2.30	69.09−78.90a
	NC	120	00.00 ± 00.00	00.00−00.00
Beauveria	$1X10^6$	90	80.66±1.76	76.74−84.60a
	$1X10^7$	90	93.33±1.76	89.40−97.26 b
	$1X10^8$	90	97.00 ±1.76	91.51−100.99b
	PC	90	79.50±1.76	74.25−85.75a
	NC	90	00.00±00	00.00−00.00

PC=Positive control, NC =negative control

Means sharing a letter in the group label are not significantly different at the 5% level

Observations of the present study indicated the presence of significant differences in mortality mean ($F = 6.58$, $P = 0.0029$) for *M. anisopliae* and ($F = 704.52$, $P = 0.0000$) for *B. bissiana* between concentrations, depending on one-way ANOVA analysis (Table 14). For *M. anisopliea*, based on pair-wise comparisons of mean (Tukey test), all concentration levels showed a significant of mortality mean when compared to sterilized distilled water (negative control). Likewise, there were statistical significances in the mortality mean of $1x10^7$ conidia/ml compared to $1x10^6$ conidia/ml and PC at $P = 0.003$ and $P = 0.002$, respectively. Regarding $1x10^8$ conidia/ml, there were also a significant difference in mortality mean compared to $1x10^6$ conidia/ml and PC at $p = 0.004$ and $p = 0.000$, respectively.

Table 14 . ANOVA results regarding the in vitro tick mean mortality

Fungal genus	Sources	SS	DF	F	P- Value
Metarhizium	Between groups	13559.2	4	6.58	0.0029
	Within groups	7725.75	15		
Total		21284.95	19		
Beauveria	Between groups	19069.7333	4	704.52	0.0000
	Within groups	62	10		
Total		19042.4	14		

SS= Sum of Square, DF= Degree of Freedom=F-statics

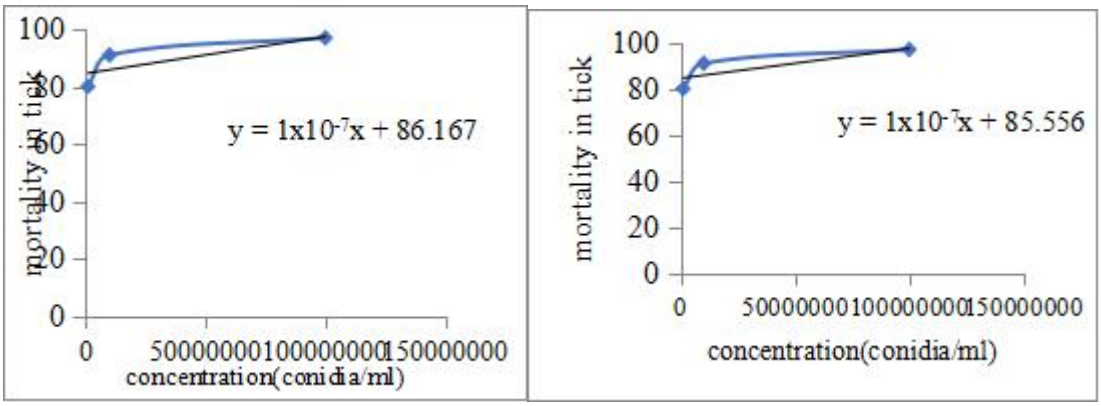
The pathogenicity of was confirmied by growing of conidia on the ticks cadavers after killing (Fig 7).

Figure 7: EPF growth on the tick cadavers

Results of the study demonstrated the lethal concentration which kills 50% of the exposed *A. variegatum* ticks (LC_{50}) for Metarhizium was while the concertation which kills 99% (LC_{99}) of treated ticks was. Likewise, for Beauveria LC_{50} and LC_{99} were calculated as and, respectively (Table 14) (Fig 8).

Table 15: Lethal concentrations (LC_{50} and LC_{99})of EPF against *A. variegatum*

Ticks	Fungal	LC_{50}	%95 CI	LC_{99}	%95CI	R^2
A. Variegatum	Metarhiz	$1x10^4$	$1x10^3$–$1x10^8$	$1x10^9$	$1x10^8$–$1X10^{13}$	99.35
	Beauveria	$1x10^4$	$1x10^3$–$1x10^6$	$1x10^9$	$1x10^7$–$1x10^{11}$	98.68



(A) (B)

Figure 8: Regression line of mean mortality of *M. anisopliea* (A) and *B.bissiana* (B) in tick.

5. DISCUSSION

Ticks are important ectoparasites of livestock because they are responsible for enormous economic losses due to their direct negative impacts and indirectly their ability to transmit tick-borne diseases. The use of chemical acaricides such as Diaznon, Amitraz and Deltametrin is the most common method of tick control in Ethiopia. However, these chemicals developed resistance due to improper application without proper training and their use over a long period of time. In addition, these chemicals have a negative impact on the environment by killing non-targated insects and causing air pollution that negatively affects the public health. Moreover, these acaricides can cause residues in animal products such as meat and milk, which pose health-threatening diseases (Msangi, 2022).

In the present study KAP was used for assessment of the knowledge, attitude and practices of animal owners regarding ticks and tick-borne diseases control in the study area. The findings of the present study in which all the respondents replied that they were aware of ticks. This is not surprising since the respondents were farmers whose livelihoods are closely linked to animals, pastures and forests where ticks are commonly infesting animals. The observation is in line with the earlier study by Kemal (2016) conducted in Mekele regional state Ethiopia. On the contrary, the results of the study reported by Namgyal *et al*. (2021) stated that more than half of respondents did not know how cattle are infested and owners don't know the sources of ticks are the environments. Majority of respondents of the current study ranked tick infestation as the 1st negative impacts on their animal health in the area. This is most probably suggesting the fact that the study area is very favorable for the survive and development of different tick stages which is associated with high constraints and negative impacts on animals of the present study area as has been suggested previously ((Alanr, 2011).

The result of the questionnaire survey of the present study showed that cattle are the species mostly affected by tick infestations than all the other animal species. This agrees with previous report by Yessinou *et al* (2018) in Benin who suggested that the ability to attract ticks is directly related to genetic factors and indirectly to environmental factors and herd management. Furthermore, Kemal (2016) reported

cattle are the most preferred livestock for tick infestation and are more commonly affected by ticks than all the other species of animals.

The findings of the questionnaire survey of the present study revealed that the indigenous breeds of cattle are generally resistant to tick infestation. This is consistent with previous report by Namgyal (2021) who suggested that indigenous breeds of cattle are considered to be highly resistant to ticks, and they are reared with minimum tick control which helps them to develop their innate immunity against ticks.

The finding of the questionnaire survey of the present study that tick infestation is most favored at the beginning of rain season followed by the mid and end of rain season with lower prevalence at the dry season repoted by the majority of respondents is in agreement with the observation of the previous study in Jimma Zone by Abebaw (2004) and Teshale and Bekele (2004) and Kemal (2016) in Borena pastoral area who recorded similar findings. The finding of the questionnaire survey of the present study that only 35% of the respondents know tick-borne diseases transmitted by ticks while the remaining 65% do not know tick-borne diseases most probably indicates that impacts of ticks has been not well understood by the community in the present the study area.

The finding of the questionnaire survey of the present study indicated that younger animals are less infested than adult and older animals by tick and it is agreement with previous report by Yessinou (2018) who suggested that the management of young cattle can contribute to the reduction of infestation. Young animals kept in a stall, reducing the risk of their exposure to ticks in pastures. The questionnaire survey of the present study indicated that male animals are more affected by ticks as compared to female animals which are most probably attributed to the difference by the stimuli and the tick tropism.

The amount of carbon dioxide (CO_2) emitted is the first determining factor in the detection of the presence of the host by the ticks. Since this quantity is proportional to the size of the host, male bovines emit much more gas than the female bovine animals, which explains the higher tick infestation load in male animals. However, on the other hand this observation contradicts the previous report by Chartier *et al.* (2000) and Gharbi and Darghouth (2014) who indicated higher tick infestation in female cattle

than the male cattle. It would be beneficial to take into account all these factors in the selection of breeds to reduce the damage by the ticks to infested animals.

The finding of the questionnaire survey of the present study that the color of the animals had no influence on the tick infestation is in agreement with the earlier report by Nwachukwu *et al*. (2020) who argued that cattle coat color had no significant impact on tick distribution, although genotypes with light coat color tended to be more infested with ticks. However, this observation contradicts the previous report by Okwuonu *et al.* (2021), who indicate that the color of livestock plays an important role in predicting the distribution of ticks on the host especially black and brown animals are more affected by ticks than the other coat color of animals.

The finding of the questionnaire survey of the present study that majority of respondents are aware of the negative effects of tick infestation and that they associate with weight loss due to blood sucking, loss of production and skin damage is consistent with the earlier findings of Kemal (2016), who indicated that most respondents attributed the impact to deterioration in body condition, teat injuries and skin damage rather than other economically important tick-borne diseases. The finding of the questionnaire survey of the present study that majority of the respondents had never heard of certain names of TBDs in cattle is in agreement with previous report by Zannou *et al.* (2021) who reported that herd owners in Burkina Faso and Benin had very little knowledge about tick-borne diseases and the problems associated with them . This could be attributed to the lack of awareness programs on ticks and TBDs among animal owners in recent years due to limited resources and other interrelated factors.

According to the finding of the questionnaire survey of the present study, more than half of the respondents aware that ticks may bite humans. However, on the contrary other respondents didn't have awareness about human tick-borne diseases which is most probably attributed to the lack of awareness programs on ticks and TBDs among animal owners in the area. This is despite the fact that tick-borne pathogens including spotted fever rickettsia was reported as the most common vector-borne pathogen second to malaria is responsible for causing systemic febrile illness among most sub-Saharan Africans (Freedman *et al.,* 2006). In this study, the majority of respondents responded that animals encountered ticks from grazing areas. Respondents assumed

that ticks hide rear or inside shaded areas in the grass, which is consistent with the life cycle of hardest ticks, particularly two- and three-host ticks as suggested before (Walker *et al* 2003).

The results of the questionnaire survey of the present study demonstrated that ivermectin was the most commonly preferred acaricide followed by amitraz and diaznon to ontrol tick infestation is in line with several previous reports by Tesfaye and Abate (2023). The respondents indicated that the preference for an acaricide was determined by the effectiveness, price, and availability of the aacricdes. However, on the other hand studies by Kenyan (Mugamb *et al.,* 2012) and Ugandan (Vudriko *et al.,* 2018) researchers found that amitraz group of acaricides was the most popular treatment for tick control. Mugambi *et al.* (2012) indicated that farmers preferred certain acaricides based on their effectiveness. In the current study area, private veterinary pharmacies were the main supplier of acaricides, which is consistent with the findings of Tesfaye and Abate (2023) in southern Ethiopia.

The results of the questionnaire survey of the present study demonstrated that 92.8% owners themselves were responsible for applying acaricides for tick control (except ivermectin) which most probably suggests the lack of qualified professionals (veterinarians and animal health specialists) particularly in rural community. However, on the other hand, previous studies by Peeling and Holden (2004) indicate that 82%, 88% and 71% of respondents from Kenya, the Philippines and Tanzania preferred CAHWs and veterinarians, respectively.

The results of the questionnaire survey of the present study demonstrated that all respondents did not gain any training on the use of the acaricides is in line with the previous report by Tesfaye and Abate (2023). This is most probably attributed to the lack of specialists with the necessary training or the owner's unwillingness to learn how to use acaricides correctly. However, on the other hand Mugabi *et al*. (2010) from central Uganda hypothesized that the majority of farmers surveyed were aware of the correct acaricide dilutions as recommended by manufacturers but ignored them. Furthermore, according to Mugabi *et al.* (2010) and Adehan *et al.* (2018) from Uganda and Benin respectively, farmers occasionally produce their own cost-effective dilution of acaricides by mixing up different acaricide groups, which is thought to be more effective.

The results of the questionnaire survey of the present study demonstrated that hand sprayer was the only option for administration of acaricides to animals with tick infestations is in agreement with earlier report by Tesfaye and Abate (2023) who indicated hand spraying was the most common method for small holder farmers to apply acaricides to their herd. This is most probably due to the high cost of additional acaricide spray techniques, including dip washing, spray racing, and powered pumps. However, on the contrary, the use of hand treatment and spray racing has been identified as the most typical and economical method of acaricide application in Bhutan (Namgyal *et al.,* 2021) and southwestern Uganda (Mbatidd *et al.,* 2021).

The results of the questionnaire survey of the present study demonstrated that majority of participants (72.22%) had knowledge regarding the impact of acaricides on both the environment and human health in general. This observation is in line with previous report by De Meneghi *et al*. (2016) who pointed out that the use of acaricidal chemicals has drawbacks that extend beyond their high direct costs. These include the selection of resistant tick populations, the possibility of compromising enzootic stability and production losses as a result of animal handling and withdrawal period, the toxicity and environmental impact on public health, and so on.

The results of the questionnaire survey of the present study demonstrated that 91.7% of respondents know that resistance to acaricides had developed in ticks which were due to the use of the chemicals in the area for a long period of time, application by an untrained person, handling methods of the chemicals and inappropriate usage of equipments. This finding is consistent with the previous report by Tesfaye and Abate (2023), who found that the most widespread under or overconcentration and frequent use of organochlorine and organophosphate compounds likely favored the development of resistance against ticks in Ethiopia. Likewise, Vudriko *et al.* (2016) in Uganda reported 93.5% resistance to at least one class of acaricide in larval population of ticks which was due to the prolonged use of acaricides over a long period of time without appropriate dose calculation.

The results of the questionnaire survey of the present study demonstrated that to reverse the problem of tick resistance against acaricides 67.2% of respondents preferred changing existing chemicals (searching for alternatives), followed by 13.3%

who preferred using ivermectin; about 7% said doubling the concentration of existing acaricides; 6.1% reported increasing frequency of application; while the rest 6.1% of respondents failed to give their suggestions. This observation suggests that respondents of the study area had good knowledge of tick resistance to acaricides, which is now a major problem worldwide. Recently biological controls against ticks using entomopathogenic fungi have received great attention (Hedimbi *et al.,* 2011).

To overcome the recently increasing challenges of tick resistance against acaricides, the introduction of environmentally friendly methods of tick control using entomopathogenic fungi (EPF), is considered a worthwhile alternative. EPF are promising alternatives for tick control due to their lack of fungal resistance and environmental safety (Fernandes *et al.,* 2010).

In the current study, EPF was isolated by using the two methods of artificial selective media and Galleria bait. The present study showed a total of 17 (21.2%) fungal species belonging to Metarhizum and Beauveria species were isolated based on their macro- and micro-morphological characterization. The study showed Metarhizium species were with higher prevalence than the Beauveris species which is in line with previous report by Mekonnen *et al.* (2024) who suggested the distribution of Metarhizium was higher in the forest habitat than Beauveria species. Likewise, Popowska Nowak *et al*. (2016) reported higher prevalence of *M. anisopliae* and *I. fumosorosea* (*Cordyceps fumosorosea*) species of EPF from the soils of several-year-old forest nurseries, especially in the spring time in various regions in Poland which was associated with the higher humidity of the soil environment. However, on the other hand Gebremariam *et al*. (2021) reported contradicting finding in which slightly higher prevalence of *Beauveria bassiana* was encountered as compared to various others EPF isolated from 183 soil samples using the insect bait method.

Findings of the present study demonstrated higher isolates (82%) of EPF in soil samples collected from forests than soils from grazing lands (12%) and soil from lakesides (2%) which is consistent with Yilma *et al.* (2019), who reported higher frequency of 64.6% of Beauveria and Metarhizium isolates in forest soils compared to 28.1% of isolates detected in farm land soils. Similarly, Gebremariam *et al.* (2021) reported higher 92.5% EPF in forest soils while only 35% in cultivated land soils. This is attributed to the fact that forest habitat is thought to provide a conducive

environment for the fungus by protecting fungi from direct contact with UV light, containing a variety of insect hosts, providing undisturbed soil, having appropriate ambient temperature and humidity, and acting as a barrier to dispersing fungal spores by winds.

Findings of the present study indicate that the distribution of both Metarhizium species and Beauveria species was higher in the Burayu sampling soils than in the Bishoftu sampling soils. This is linked to agro-ecological factors like altitude, climatic conditions, and soil types which can influence the distribution of fungi (Moragan *et al.* 2006). Burayu is found at a high altitude and contains a loam soil type that contains the moisture required for fungal viability While Bishoftu is located on midland and lowland agro ecology mostly vertisole types of soil, which is characterized by having low moisture contents and crack during the dry season. In support of this our observation Mekonnen *et al.* (2024) stated that the occurrence of EPF was strongly correlated to soil characteristics with high organic matter contents and an elevation altitude ranging from 1800 to 2600 meters, even though fungi were occurred at a wide range of altitudes (Moragan *et al.* 2006).

Findings of the present study showed that statistically significantly higher EPF were isolated using the *Galleria mellonella* biat methods 14(82.3%) than the artificial selective media method 3(17.6%) which is in line with previous report by Keyser *et al.* (2015) who indicated out of an overall isolation of 132 *Metarhizium* spp, 118 isolates were obtained using insect baiting methods while only 14 isolates were obtained by selective media. Similarly, Correa *et al.* (2022) reported higher percentage of positive samples has been isolated using insect baits method as compared to artificial selective media for the isolation of EPF. Imoulin (2011) stated that insect baiting is a more efficient methodology for EPF isolation over culturing soil suspensions on selective media and added that selective medium can only be viewed as a semi-quantitative method for EPF isolation as it may provide a false picture of fungal diversity and density, leading to a biased view of many microbial systems. Meyling (2007) also suggested over growth of soil opportunistic fungi and small soil sample (1gram) has the risk of not sampling the fungus due to EPF usually clumping in the soil affect the efficiency of artificial selective media.

Results of the current study demonstrated the acaricidial efficacy of both Metarhizium and Beauveria EPF against the economically most important *Amblyomma varigatum* ticks. The in vitro efficacy evaluation of EPF against *A. varigatum* ticks disclosed the presence of potential to control the challenging negative impacts of tick infestation in the country and might be the most promising alternative for livestock owners in the future. There is also ample evidence that indicate escalation of tick resistance against acaricides currently in Africa (Evans *et al.,* 224).

The observation of higher overall mortality percentage in *A. variegatum* ticks with higher concentrations of EPF ($1x10^7$ and $1x10^8$) conidia/ml 90.9% than the commercial acaricide Amitraz 76.8% in the current study is in agreement with previous study by Tesfaye and Abate (2023) who reported only 73.15% mortality using commercial acaricides while very high 90.9% percentage oviposition control in Borana pastoral areas (Eshetu *et al.,* 2013). Tibebu and Assefa (2023) argued that this is most probably attributed to the development of resistance against *A. varigatum* tick species against the commonly in use acaricides in the study area as well as other parts of Ethiopia sooner than later. Similarly, Asha and Eshetu (2015) reported an average efficacy of 65.3% for diazinon in Wolaita and Dawuro zones which was even lower than the 76.8% for amitraz in the current study. In support of this our study lower in vitro efficacies of amitraz against *Rh. microplus,* have been already reported in many countries including 14.2% and 56.3% in Colombia (Lopez *et al*., 2014), 30.95% in Brazil (Campos and Oliveira, 2005), and 48.4% in the Brazilian Southwestern Amazon (Brito *et al*., 2011).

The result of the present study confirmed the presence of good in vitro acaricidal efficacy of EPF against the *A. variegatum.* Accordingly, the mortality of adult ticks by *Metarhium anisopliae* ranged between 50% and 100% for the three ($1x10^6$, $1x10^7$, and $1x10^8$) conidia/ml concentrations 15 days after inoculation. In support of our study, Hedimbi *et al.* (2011) also observed that *M. anisopliae* was capable of causing mortalities at $1x10^8$ conidia/ml concentration to various developmental stages of, *A.variegatum, Rh. apendiculatus* and *Rh evertsi* in both water and oil formulations.

Another study conducted in Iran by Pirali *et al.* (2007) revealed that the highest mortality of engorged females of *Boophilus annulatus* 15 days after inoculation was 100% for *Metarhizium anisopliae* strain DEMI001 at the concentration of 10^7

onidia/ml. Likewise, Cafarchia *et al.* (2015) showed that the mortality for all developmental stages of *Rh. sanguineus s.l.* was 100% within 15 days at 10^7 conidia/ml concentration. In addition, Kaaya and Hedimbi (2012) reported high mortality 100% in both *Rh (B).appendiculatus* and *Amblyomma variegatum* tick species using aqueous and oil formulations of *M. anisopliae* ($1x10^8$) conidia/ml especially in larvae. However, on the other hand study by Diaz *et al.* (2019) reported low reduction (<45%) using *M. anisopliae* conidia in water formulations containing Tween80 for the control of *Rh. (B.) microplus* in tick populations feeding on cattle. These difference in efficacy might be attributed to various factors such as animal hair preventing fungal attachment to the ticks, UV light affecting the viability of EPF, inappropriate humidity, and application technique might be affecting the fungal effectiveness.

Similarly, *Beauveria bissiana* yielded a promising result with mortality mean which was varied depending on fungal concentration levels. Low mortality mean of 80.66% at $1x10^6$conidia/ml and high mortality mean of 93.33% and 97%) atrespective concentration of $1x10^7$ and $1x10^8$ conidia/ml was recorded 15 days after inoculation. This is in line with previous study by Gomathinayagam (2002) who reported high mortality in *Amblyom mericanum* exposed to con dia concentrations at 3.04 x 10^7 and 3.04 x 10^8. Similarly,Kaaya and Hedimbi (2012) also stated that *Beauveria bissiana* had 100% mortality in *Rh. (B.). appendiculatus* and *A. variegatum* after applying a fungal concentration of $1x10^8$ to potted grass in the field. In contrast to findings of the present study, Aboelhadid (2018) reported *B. bassiana* did not show a lethal effect on adult female *Rh. annulatus* at any concentration. Furthermore, Kaaya *et al.* (1996) reported low mortality of 30% and 37% in *Rh. appendiculatus* and *A. variegatum,* respectively, after application of *B. bassiana*. This variation in efficacy possibly indicates differences in application methods, differences in fungal strain potency, formulation methods, and the low viability of the fungus at the time as has been suggested before (Hedimbi *et al.* (2011).

Experimental observations of the present study demonstrated that all selected isolates of EPF tested in vitro efficacy against adults of *A. variegatum* ticks possessed lethal effect. However, statistically significant difference in the efficacy of EPF of either species of fungal isolates was not observed. Although slightly higher average

mortality was recorded in ticks treated with *M. anisopliae* than those ticks treated with *B. bissiana*, which is in line with previous study done by Murigu *et al, (*2016), Who reported that Isolatesof *M. anisopliae* was generally more pathogenic than those of *B bassiana* and there was variability between the fungal isolates.

Experimental observation revealed that mean mortality in tick increased with increasing concentration level and duration of exposure time. This observation is consistent with the previous study by Kirkland *et al*. (2004), who reported that dose-dependent mortality was found in unfed adult and nymphal *Rh. sanguineus* and *I. scapularis,* with mortality limited compared to *D. variabilis* ticks by the use of the same fungal species, harvested from either agar plates or liquid media and washed in sterile dH_2O containing 0.01% Tween 20.

For *M.anisopliea*, based on pair-wise comparisons of mean (Tukey test), all concentration levels showed a significant of mortality mean when compared to sterilized distilled water (negative control). Likewise, there were statistical significances of $1x10^7$ conidia/ml compared to $1x10^6$ conidia/ml and PC at P = 0.003 and P = 0.002, respectively. Regarding $1x10^8$ conidia/ml, there were also a significant difference compared to $1x10^6$ conidia/ml and PC at p = 0.004 and p = 0.000, respectively.

Similarly, in ticks treated with *B. bissiana* isolates, all concentrations showed statistically significant differences in mortality in ticks compared to the negative control. However, only some isolates showed statistically significance difference in their mortality mean compared to the positive control (Amitraz 12.5% concentration). Accordingly, the $1x10^7$ conidia/ml concentration showed a significance difference with $1x10^6$ conidia/ml at P = 0.001 and with PC at P=0.000. Likewise, highly significant differences at $1x10^8$ conidia/ml compare to the $1x10^6$ conidia/ml and positive control (Amitraz) at P = 0.000 was observed.

The probit regression test was used to determine the LC_{50} and LC_{99} of fungal concentration. Accordingly, the range of LC_{50} was $1x10^3$−$1x10^8$ for *M. anisolpiae* and $1x10^3$-$1x10^6$ conidia/ml for *B. bissiana.* This observation is in agreement with the previous study by Fernandes and Bittencourt (20012) who recorded LC_{99} value between $1x10^8$ and $1x10^{13}$ for *M. anisopliae* and between $1x10^7$ and $1x10^{11}$ for *B.*

bissiana on tick larval mortality on the same fungal species. However, results are less compared with other reaserch reported done by Msangi (2020) in Tazanya on other species of EPF against different stages of Amblyomma tick species, LC_{50} valus was $2{,}2x\ 10^3{-}6.0\ x\ 10^3$ and $1.0\ x\ 10^6{-}3.0\ x\ 10^6$ for LC_{99}. Which is suggested the difference is dueto difference in EPF and tick species as well as geographical location (Cafarchia *et al.,* 2015).

The fundamental advantages associated with the mode of infection of EPF as compared to commercial acaricides lie in their ability to use different mechanisms to colonize and kill ticks. EPF utilize enzymatic, toxicological and mechanical invasion systems, suggesting that it is difficult for ticks to develop resistance to EPF. In addition, they are known to be able to control almost all phases of the arthropod life cycle, which is another major advantage as a member of pest control programs (Srinivasan *et al.,* 2019). Beys-da-Silva *et al.* (2020) also suggested the infection mode of EPF in ticks involves (1) recognition of the susceptible host; (2) adhesion of conidia and germination to the host cuticle; (3) development of specific structures (germ tube and appressorium) ;(4) penetration through the host cuticle; (5) heavy fungal growth and death of the host; and (6) production of conidia following passage of hyphae through the host cuticle. The limitations of the present study include some farmers were reluctant to give their ideas regarding tick control activities in the area and also the small number of female farmers as a house hold leader in Ethiopia contest might have affected the quality of the data generated from the respondents.

6. CONCLUSION AND RECOMMENDATIONS

The data generated from present study demonstrated that tick infestation is one of the major problems of animals in the study areas. Findings of the study suggest the decline in efficacy of commercial acaricides in controlling tick infestations. Lack of regular training on KAP of animal owners and the community on ticks and tick-borne pathogens of economic and public health importance would reduce the negative impacts on both the veterinary and medical issues in Ethiopia. Observations of the present study attest that statistically significantly higher EPF were isolated using the *Galleria mellonella* biat methods 14(82.3%) than the artificial selective media method 3(17.6%). Results of the current study demonstrated the acaricidial efficacy of both Metarhizium and Beauveria entomopathogenic fungi against the economically most important *Amblyomma varigatum* ticks. The in vitro efficacy evaluation of EPF against *A. varigatum* ticks disclosed the presence of potential to control the challenging negative impacts of tick infestation in the country and might be the most promising alternative for livestock owners in the future. Thus, EPF has the potential to be an alternative to these chemical acaricides as it protects the environment from the accumulation of chemical residues other negative impacts. The present study is the first in the country to control investigate EPF against ticks of economic and public importance. Based on the above conclusion, the following recommendations are forwarded:

- Awareness campaigns should be targeted at minimizing or preventing misconduct and health hazards due to knowledge, attitude and practice gaps that lead to ineffective use of acaricides, ticks and tick-borne pathogens in Ethiopia.
- Further in detail studies using molecular and other high-quality methods on in vivo and in vitro efficacy of EPF should be conducted in the future.
- Further in detail studies using molecular and other high quality methods development stage of tick species and tick-borne pathogens should be conducted in the future.

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8. APPENDICES

Appendix 1: Questionnaire survey format

Thisquestionnaire format is designed to gather information on the Efficacy of Entomopathogenic fungi against *Amblyomma Variegatum* Ticks and their control practices in and around Bishoftu, Central Oromia, Ethiopia After introducing the scope and the objectives of the study, selected farmers were asked for their full consent to participate in the interview. Only, those willing to participate were considered for the questionnaire survey. All information each respondent provides and his/her name was remaining confidential.

Personal information

Respondent Name.....

District: .....PA: .....

Village: .....GPS.....

Respondent No---------- Gender: Male------- Female--------

Age (years) (circle the correct number) a) Below 25 years b) 26-30 c) 31-40 d).41-50 e). 51-60 f). Above 60 years

Educational level: a) Illiterate b) Local school c) Elementary d) High School e) College Diploma f) Degree

1. Livestock management and farming system

1.1. Which livestock species do you keep? A) Cattle b) sheep c) Goat d) Equines e) poultry f) mixed

1.2. Which breed of cattle do you have? A. zebu B. exotic cross C. both

1.3. For what purpose do you keep cattle? a) Milk b) Fattening c) Draught d) Milk and Draught

1.4. What is the management system of cattle? a) Extensive b) semi-intensive c) intensive

1.5. Where do your cattle graze (if not intensive) ----------

2. Knowledge of Livestock keepers towards tick control

2.1. Do you know ticks? a) Yes b) No

2.2. What is the rank of ticks in terms of negative effects on animals? a) 1^{st} b) 2^{nd} c) 3^{rd} d) 4^{th} e) 5^{th} f) $>5^{th}$

2.3. Which livestock species is affected mostly by Tick? a) Cattle b) sheep. c) Goats. d) Equine e) poultry f) All

2.4. Do you know the names of ticks species which affect different spps of animals in your own language?

a). Yes b). No

2.5. Breed of animals comparatively more susceptible to the tick infestation in your locality

a) Indigenous breed b) Cross breed c) Exotic breed d) All are equally infested

2.6. In which season of the year Tick infestation is more common? a) At the end of rainy season b) At the beginning of rainy season c) in mid of rainy season d) At the dry season

2.7. Which groups of cattle are more affected with tick a) young animals b) Adult c) Older d) All

2.8. Which sex category of animals is more affected? a) Female b) Male c) equal d) don't know

2.9. Which Color of animals more susceptible tick infestation in your locality? a) Black animals b) white animals c) grey animals d) red animals e) mixed f) all

2.10. What is the impact of tick infestation on general animal health and production? a) Weight loss b) Production loss c) Teat damage d) Hide damage e) Tick born disease

2.11. Do you know tick bite human

A). yes b) no c) not known

2.12. Does tick bite transmit disease to human? A. Yes B. No C. not Known

2.13. Where are the sources of ticks (from where do cattle encounter the ticks) a) vegetation b) grazing areas c) areas covered with crops d) water sources e) every where

3. Attitude and practices of Livestock keepers on Tick control methods.

3.1. What Mechanisms do you use to control ticks? a) Manual removal b) Using Acaricides c) by injection d) Medicinal plants

3.2. Which Acaricidal drugs do you use to tick control? a) Amitraz b) Diaznone c) Ivermectine d) Deltametrine

3.3. Which Chemicals do you prefer frequently for the tick control and why? ----------

3.4. From where do you get chemicals? a) Veterinary clinics b) private drug shops c) open market and illegal sources

3.5. Who is Responsible for acaricidal application? a) Owners b) Veterinarians c) CAHWs d) All

3.6. If you perform acaricidal application, did you get training on how to use it? a)Yes b) No

3.7. Method of application a) Dipping b) spraying c) pour on d) hand picking

3.8. Equipment used for application a) knap sack sprayer b) bucket/foot pump c)hand sprayer d) spray race e)scrubbing cloth

3.9. Do you know the negative impacts of acaricides on human health and environment? a)Yes b) No

3.10. If the question no 3.7 is yes, list the impacts----------

3.11. Is there tick resistance to acaricides? a) Yes b) No

3.12. What Measures should be taken to avert tick resistance: a) find other alternative b) Double concentration of acaricide c) Increase the frequency of spraying d)Triple acaricide concentration e) Use ivermectine f) not applicable

Thank you!

Appendix 2 Selective media for isolation of entomopathogenic fungi

Selective medium for *Metarhizium spp*

Suspend 32.5 gram SDA (Sabouraud Dextrose Agar) in 500 ml distilled water in a blue cap bottle.

Mix the medium and mark the blue cap bottle with autoclave tape.

Autoclave the medium for 20 min at 120 C 20 bar. (Remember that the lid of the blue cap bottle has to be loose during autoclaving)

Cool the medium after autoclaving to approx. 60 C and add:

500 ml Chloramphenicol (inhibits bacterial growth)

500 ml Streptomycin sulphate (antibiotic, inhibits bacteria)

Invert the bottle gently and pour the plates.

Appendix 3: Isolation using insect baits

1. Use surface-disinfected *G. mellonella (*from the fourth stage) and Immerse the larvae into 0.5% sodium hypochlorite for 1 min for sterilization. Wash the larvae twice using sterile water.

2. Plastic pots are used to assemble the baits. A 250 g of collected soil will be added to each plastic pot (98 mm width x 47 mm height x 142 mm length) and deposit five larvae per plastic pot and the pots will be stored at 25 ± 1 C and relative humidity ≥ 80% in the dark. 10 small holes will be drilled (2 mm in diameter) in the pot lids to allow ventilation. A sharp heated iron device will be used to drill the holes.

3. Homogenize the soil every other day to allow maximum contact of larvae with soil. Moisture is important to support the fungal infection of larvae. To maintain moisture in the soil, spray sterile distilled water on the soil surface will be done whenever necessary.

4. The pots will be analyzed by daily seeking dead insects. The remaining larvae in the colony will be observed daily for invertebrate pathological signs to make sure the insects are not infected. As an alternative, control pots with sterile soil will be included in the study to check the health status of the insect larvae.

5. The died insects will be removed and superficially sterilize them with 1% sodium hypochlorite for 1 min then the sterilized insects will be placed in a humid chamber (relative humidity ≥ 80%) at 25 ± 1 °C for 7 days to favor the exteriorization of entomopathogenic fungi (mycosis).

6. Upon mycosis, the conidia will be harvested from the insect surface. A microbiological loop will be used to place the conidia on PDAC medium under a stereoscopic microscope. As an alternative, the whole infected larvae will be placed on the PDAC medium. The culture plates will be incubated in a climate chamber at 25 ± 1 °C and relative humidity ≥ 80%.

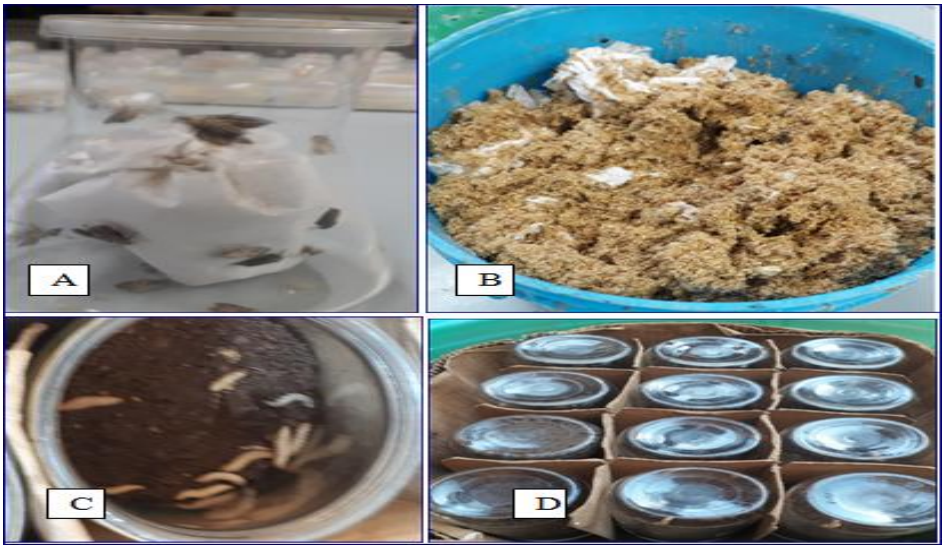
7. Observe the macromorphology and micromorphology of the fungal colonies on the plates to confirm the identity of EPF. Culturing on PDAC will be repeated until pure fungal colonies are obtained.

Appendix 4: Selective media preparation process

Pictures of weighing 10-gram soil (A), putting in to the test tube(B) ,Pouring 100µl solution suspentions on The SDA media(C) and ready for the incubtion(D)

Appendix 5 :Rearing Galleria process

Rearing galleria in the jar (A), Plastic box with galleria larva and food ingredient (B), putting the galleria larvae in the soil (C) then turning down (D) the jar to facilitate the contact b/n galleria larvae and fungi in the soil



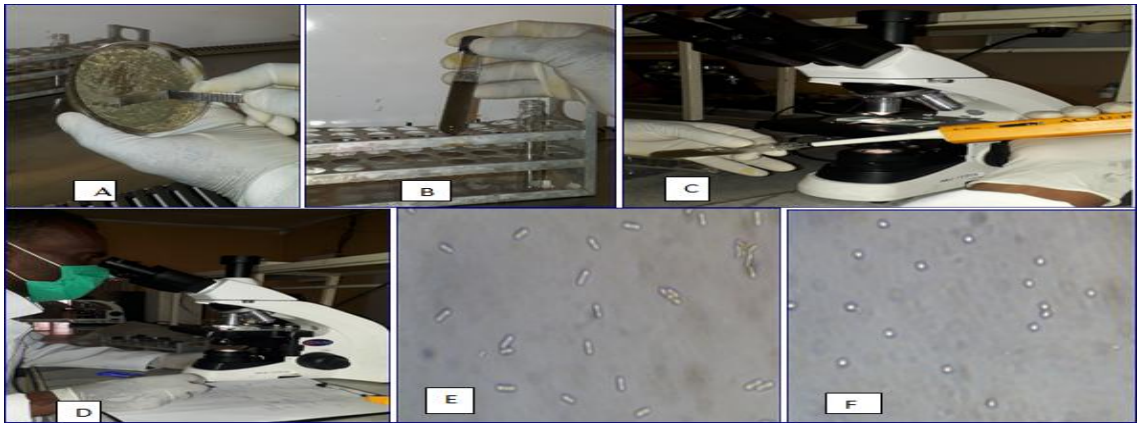
Appendix 6: Isolation of EPF by Galleria bait methods

Died of the galleria larvae after ten days (A), *M. anisopliae* spores on insect cadaver (B), *B. bissiana* on the insect cadaver (C), colony of *M. anisopliae* on SDA (D=front side, E=back side)and *B. bissiana* colony on SDA.

Appendix 7: Fungal concentration preparation process

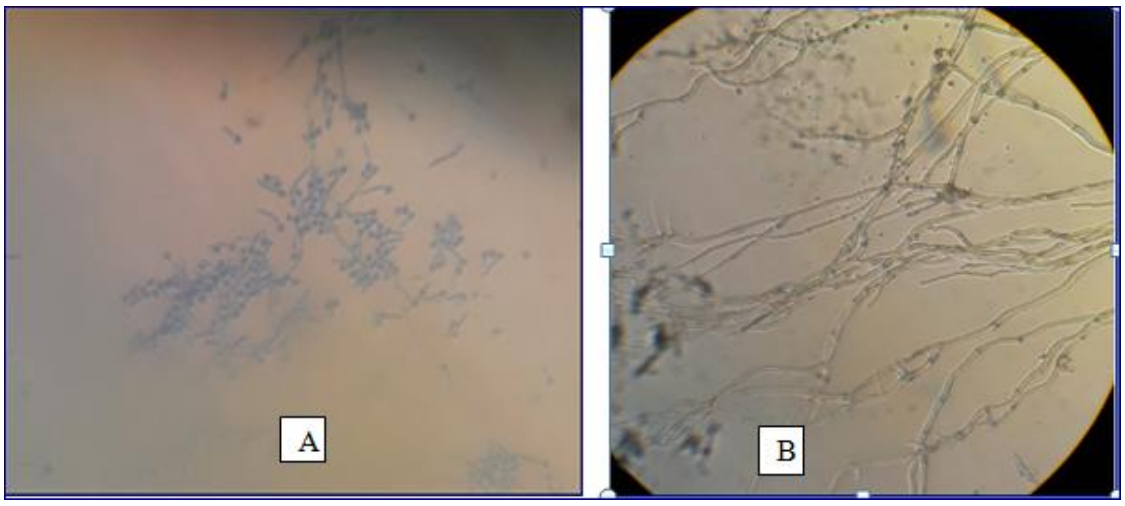
Scraping the sporolated pure colony (A) for stock solution preparation, stock solution after vorted for10 min (B), peppating fungal solution into heamatocyto

meter (C), counting the spores (D) and rode shaped M. anisopliae (E) and spherical shaped B. bissia (F).

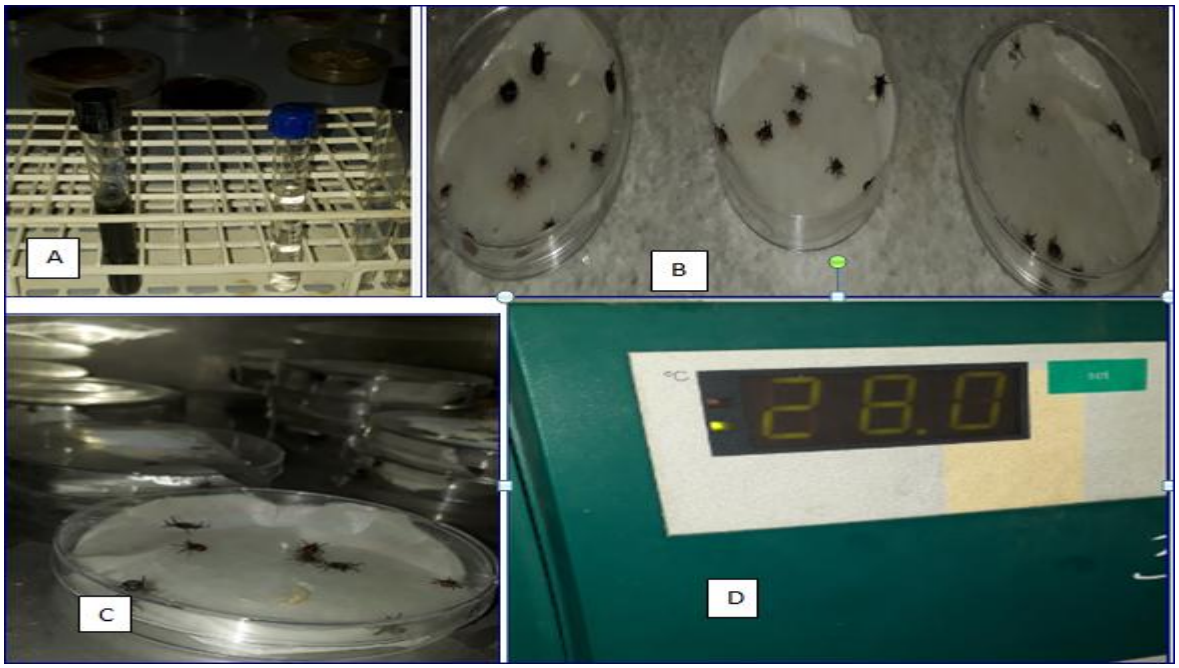


Appendix 8: Staining of fungi by Lactophenol cotton blue

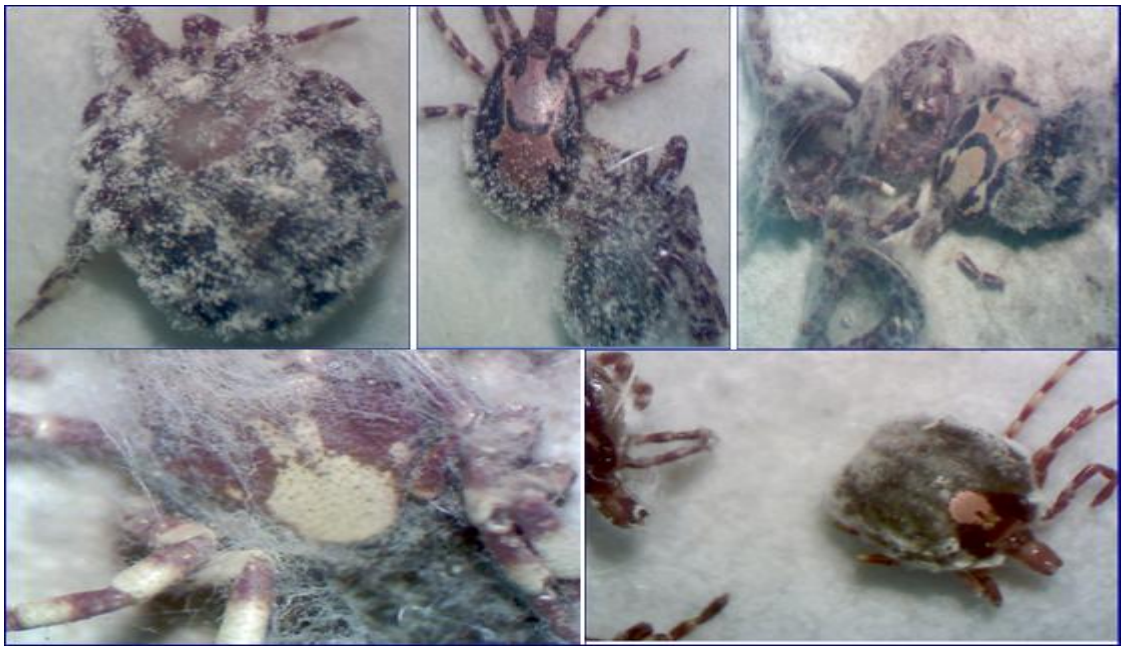
Spores and hyphal structures of Beauveria (A) and Metarhizium (B) isolates observed with compound microscope (40 x magnifications) after stained by Lactophenol cotton blue



Appendix 9: working concentration preparation and applying on ticks
working concentration prepared by Serial dilution (A), treated tick (B), Incubation of the treated tick(C) and incubater (D).



Appendix 10: Fungal growth on tick cadavers



Appendix 11: Different medium and chemicals used during the study.

Appendix 12: questioner consent form

Good morning/good afternoon!

I am MSc student at the College of Veterinary Medicine and Agriculture at Addis Abeba University and I am conducting research for my thesis on Efficacy of Entomopathogenic fungi against *Amblyomma Variegatum* Ticks and their control practices in and around Bishoftu, Central Oromia, Ethiopia, The purpose of this study is to isolate and identify entomopathogenic fungi from soil of farm land areas of the district and evaluate their efficacy against the tick under laboratory conditions. I would like to know your thoughts on the tick infestation and its control methods. I believe that this study will be very helpful in identifying the condition and the elements that contribute to it, and it will produce original data that will be a tremendous help in informing future action by the public sector and others. In light of this, it is crucial that you provide honest answers to all of the questions. Your participation in this study is entirely voluntary, and there are no personal gains or risks for you as a result. I guarantee that your response will be kept private and that any data gathered about your personal identify won't be disclosed to a third party as is standard research procedure. During the report or presentation of this study, no one will learn your identity in relation to specific questions and answers.

Respondent statement: I have understood the above statements:

Yes (Agree to participate)

No (Not agree to participate)

Name of respondent: _____

Signature: _____

Date: _____

Appendix 13: Ethical clearance letter

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/15/2024

Name of Applicant: **Dereje Tsegaye Tadesse (DVM, MSc student)**

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

Title of the project: *Efficacy of Entomopathogenic fungi against ticks of cattle and their control practices in and around Bishoftu, central Oromia-Ethiopia*

Date of application:	December, 2023
Nature of the project:	Laboratory test and questionnaire survey
Target animal species:	No animal Use
Number of animals involved:	None
Study area:	CVMA-Bishoftu, Ethiopia

Minutes No. and date of review: **VM/ERC/02/15/024, 26/03/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Dereje Tsegaye Tadesse .**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

Please quote Our Ref. No. When replying

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ex 251-11-4339033 Tel. +251 114338450 P.o.x. Box)34 Bishoftu, Ethiopia