Addis Ababa University College of Veterinary Medicine and Agriculture

Thesis Title

Assessment of Histofarcin Skin test and *In vitro* Evaluation of Effects of Selected Medicinal Plants on the Growth Inhibition of Mycelial form of *Histoplasma Capsulatum* var. f*arciminosum*

By

Hawi Jaleta Sofani

Department of Clinical Studies

MVSc Program in Veterinary Epidemiology

June, 2019

Ethiopia

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

Assessment of Histofarcin Skin Test and *in vitro* Evaluation of Effects of Selected Medicinal Plants on the Growth Inhibition of Mycelial form of *Histoplasma capsulatum* var. f*arciminosum*

A Thesis submitted to Addis Ababa University College of Veterinary Medicine in partial fulfilment of the requirement for the degree of master of Veterinary science in Veterinary Epidemiology

By
Hawi Jaleta

MVSc Program in veterinary Epidemiology

Advisors: Teshale Sori (DVM, MSc, PhD, Associate Professor)
Gobena Ameni (DVM, PhD, Professor)

June, 2019

Bishoftu
Ethiopia

TABLE OF CONTENTS **PAGES**

ACKNOWLEDGEMENTS

First of all, I would like to thank the almighty God for his protection and peace.

Next, my great appreciation will go to Dr. Teshale Sori for guiding and giving me his precious time and skill without any hesitation. Professor Gobena Ameni also deserves my appreciation for his provision of literature and reading materials, routine follow up and allocating a budget for this study.

My warm appreciation will be for Department of CLIS, Epidemiology program, Addis Ababa University College of Veterinary Medicine and Agriculture, for facilitating and coordinating this course.

All staffs of SPANA project, Aklilu Lema Institute of Pathobiology, Addis Ababa University College of Veterinary Medicine and Agriculture and all friends and colleagues who encouraged and supported me deserve my heartfelt appreciation.

LIST OF ABBREVIATIONS

CNS	Central Nervous System
EL	Epizootic Lymphangitis
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agricultural Organization
FAT	Fluorescent Antibody Tests
FNA	Fine Needle Aspiration
HA	Heamagglutination
HCF	*Histoplasma capsulatum var.farciminosum*
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PPLO	pleuropneumonia like organism

LIST OF FIGURES **PAGES**

LIST OF TABLES **PAGES**

LIST OF ANNEXES **PAGES**

ABSTRACT

Epizootic lymphangitis is one of the neglected infectious diseases of equines causing remarkable loss in cart horses in Ethiopia. Lack of effective treatment and appropriate diagnostic tools for screening of the disease contributed to its continued occurrence. In this study, histofarcin skin test was developed and evaluated on 10 clinically infected, 10 healthy and 10 recovered horses. The histofarcin antigen used was prepared from field isolates of *Histoplasma capsulatum var farciminosum*. The concentration of the antigen was determined using bicinchonic acid protein assay method (Pierce, BCT^{TM} protein assay kit number 23225) at Black lion teaching hospital, Addis Ababa University. Each horse was injected with 0.1mL of the antigen intradermally in the middle of their neck. The thickness of the skin was measured before injection and 24, 48 and 72 hrs post injection using Bar knight Mc Lintock (McLK- P0005) calliper. The sensitivity and specificity the skin test was 80 % and 70%, respectively. In addition, the *in vitro* growth inhibitory effects of the root of *Curcuma longa*, berry of *Phytolacca dodecandra* and leaf of *Datura Stramonium* were evaluated. For this isolates of *Histoplasma capsulatum var farciminosum* were identified from four sites (Bishoftu, Akaki, Holota, Mojo). Two fold serial dilutions of the methanol extracts of the three plants were prepared and assessed. The minimum inhibition concentration of the plants was determined by agar dilution assay at Aklilu Lema Institute of Pathobiology, Addis Ababa University. A culture media with no antifungal agent and media containing ketoconazole served as negative and positive control, respectively. The results showed that *Curcuma longa* and *Phytolacca dodecandra* had inhibitory effect at ranges of concentrations. *Curcuma longa* had lower minimum inhibitory concentrations (0.07mg/mL) than *Phytolacca dodecandra* (0.156mg/mL) where as *Datura stramonium* showed no inhibitory effect on *Histoplasma capsulatum var farciminosum*. In conclusion, this study demonstrated that histofarcin skin test is a promising diagnosing tool for screening of equine histoplasmosis. Besides, the methanol extracts of *Curcuma longa* and *Phytolacca dodecandra* showed strong *in vitro* growth inhibitory activity against *Histoplasma capsulatum var farciminosum*.

***Keywords**: Cart horses, Epizootic lymphangitis, Ethiopia, Histofarcin skin test, Medicinal Plants*

1. INTRODUCTION

Horses provide different services to the community including ploughing and transportation (Guerin *et al.,* 1992).They are mostly known for their transportation services such as household and agricultural goods especially in poorly developed countries since long years back. However, the optimum works output expected from horses and the consequent benefits gained by the society are halted by diseases. Equine histoplasmosis is among the major health problems of horses (Wilson, 1995).

Equine histoplasmosis is caused by a dimorphic saprobic fungal agent *Histoplasmacapsulatum var.farciminosum* (HCF) (*synonyms: Cryptococcus farciminosum, Zymonemafarciminosum, Histoplasma farciminosum*and *Saccharomyces farciminosus*), which exists as a yeast in animal tissues and a saprophytic mycelium in the environment. The mycelial phase prefers humid and moist environments and is thought to persist in the environment where the disease is endemic while the yeast form is pathogenic and is found in lesions (OIE, 2018). Equine histoplasmosis, also called epizootic lymphangitis (EL), is mostly a disease of horses but it sometimes affects donkeys and mules. The disease is characterised clinically by a suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis in horses (Tagesu, 2017). It is also reported in camels, cattle, dogs and experimental animals such as rabbit, mice and Guinea pigs (Lowa state University, 2009). Although the zoonotic importance of the disease is not well recognized, rare human infections by HCF have been reported (Al-Ani, 1999).

Different methods have been employed for the diagnosis of EL. These include detection of the agent from the lesions using Gram's, Giemsa or Periodic Acid-Schiff staining (Rahmeto *et al*., 2017), serological tests such as Direct and Indirect Fluorescent antibody tests, Enzyme Linked Immunosorbent Assay and Passive Heamaglutination tests (OIE, 2018).The innovative skin test (Histofarcin test), which detects cell mediated immunity, has been developed (Ameni *et al*., 2006; Gabal and khalifa, 1983). However, all of the diagnostic methods listed have their own limitations regarding sensitivity, specificity, detection of the case in its early stage and financial constraints among others. Easy, rapid and cheaper diagnostic method is needed for understanding of the epidemiology of the disease.

In densely populated areas where large numbers of horses are reared together the incidence of EL can be very high. The disease is common in cart horses in Ethiopia, affecting on average 18.8% of horses in warm and humid areas causing considerable losses to the owners and the society (Tagesu, 2017). One reason for the wide spread occurrence of EL is the absence of effective treatment and control options. Various treatment approaches have been used throughout the country but none was found effective. As a result, infected animals remain source of HCF for healthy horses. Besides, due to lack of awareness by the owners of horses, harnessing materials are being shared among them. To circumvent the situation the owners and traditional healers have been using different plants to treat EL since long time. These plants constitute promising alternatives to iodides and amphotericin-based therapies (Birhanu *et al*., 2007; Lowa state University, 2009). Screening of the plants used traditionally in the treatment of EL is an important aspect of management of the disease. That is, *in vitro* assessment of the effect of medicinal plants is a pre-requite for understanding of their pharmacodynamic property. Previous studies demonstrated promising results although their findings are variable (Negese *et al*, 2012; Fantahun, 2016).

Therefore, the objectives of this study were to assess the potential of histofarcin skin test for the detection of equine histoplasmosis and *in vitro* evaluation of effects of *C. longa*, *P. dodecandra* and *D. stramonium* on the growth inhibition of HCF.

2. LITRATURE REVIEW

2.1. Etiology

Equine Histoplasmosis is caused by the dimorphic saprobic fungal agent HCF. The filamentous mould form is found in the environment and can be cultured in conditions below 35ºC using general fungal media. When it is present in tissue or grows in cultures, using brain heart infusion agar or brain heart infusion with blood at temperatures greater than 35ºC, it grows to a yeast form, which appears as small oval buds of 2-4 μm. Macronidia are 8-15 μm and are thick walled with tubercles or projections. The micronidia are 2-4μm and are smooth walled (Lowa state University, 2009). Under experimental conditions, inhalation of 5 yeast cells resulted in 10 % mice developing infection. Ten spores administered intra-nasally resulted in a 9.2 % mortality rate in mice (Tagesu, 2017).

The yeast form of the organism is present in large numbers in well-established lesions, and appears as pleomorphic ovoid to globose structures, approximately 2–5 μm in diameter, located both extracellular and intracellular in macrophages and giant cells. Organisms are usually surrounded by a 'halo' when stained with Gram stain, haematoxylin and eosin, Giemsa, periodic acid–Schiff reaction or Gomori methenamine–silver stain (OIE, 2018). The fungus persists for months in moist and warm conditions. The organism was first demonstrated in the pus by Revolt in 1873 but the first successful isolation of the fungus was reported in 1886 in Japan (Mahendra, 2012). HCF is susceptible to 1% solutions of sodium hypochlorite, 20% phenol, 2%glutaraldehyde, isopropyl alcohol and formaldehyde. Its spores and yeast cells are inactivated when held at temperatures above 40 ºC for extended periods of time. Growth is inhibited at PH below 5 and above 10. Dry conditions also facilitate the inactivation of the spores. *Histoplasma capsulatum* is found primarily in nitrogen rich soils with a PH ranging 5-10. Moist soils at temperatures between-18 to 37 ºC can support the growth of the fungus. Organisms are known to survive excess of 10 years in soil (Lowa state University, 2009).

2.2. Epizootic Lymphangitis

Infection by HCF usually presents as a cutaneous or pulmonary form, which may be transient or chronic. There are a number of clinical forms, including asymptomatic (Tagesu, 2017), acute pulmonary, chronic pulmonary, acute disseminated, chronic disseminated, mediastinitis, meningitis, osteomyelitis, and cutaneous infection (OIE, 2018). Widespread infection (dissemination) throughout the body has resulted in death; however, dissemination usually results in chronic illness. Symptoms can appear with in 1 week and include fever, non-productive cough, myalgia, chest pain, loss of appetite and fatigue. Infection is usually asymptomatic for low level exposure or mild infections. There is no evidence that mild or asymptomatic infections can induce protective immunity. Interestingly it has been noted that previously controlled infections can become active upon impaired cellular immunity. Higher levels of exposure and more severe infections will result in symptomatic presentations like flu-like symptoms that may persist for several weeks. Visceral infection can lead to the destruction of the adrenal gland. Infection of the CNS will appear as chronic meningitis or focal brain lesions and occurs in 5-20 % of the cases. Infection sites are typically the skin and bones and will spread to the liver and spleen if left untreated. Lesion on face and trunk are common. Thirty percent of patients develop osteomyelitis. Arthritis and purulent subcutaneous abscesses can occur when the infection spreads to joints and soft tissue. The mechanisms underlying host specificity and tissue tropism remain unknown. The disease mostly occurs in three forms; cutaneous form which involves the extremities, chest wall and the neck (figure 1 A), ocular form (figure 1 C); which present as an ulcerating conjunctivitis of the palpebral conjunctiva and respiratory form (figure 1 B) as a multifocal pneumonia (OIE, 2018). The incubation period of the disease is from 3 to 12 weeks. Even if the mortality is low, the disease causes debility and inability to work because of painful lesion. Post mortem examination may reveal thickened areas of the skin fused to the underlying tissues. Enlarged and inflamed regional lymph nodes. Nodules in the skin have a thick, fibrous capsule and the affected lymphatic vessels are usually thickened or distended. The lungs, spleen, liver, testes and other internal organs may also contain nodules and abscesses (Lowa state University, 2009).

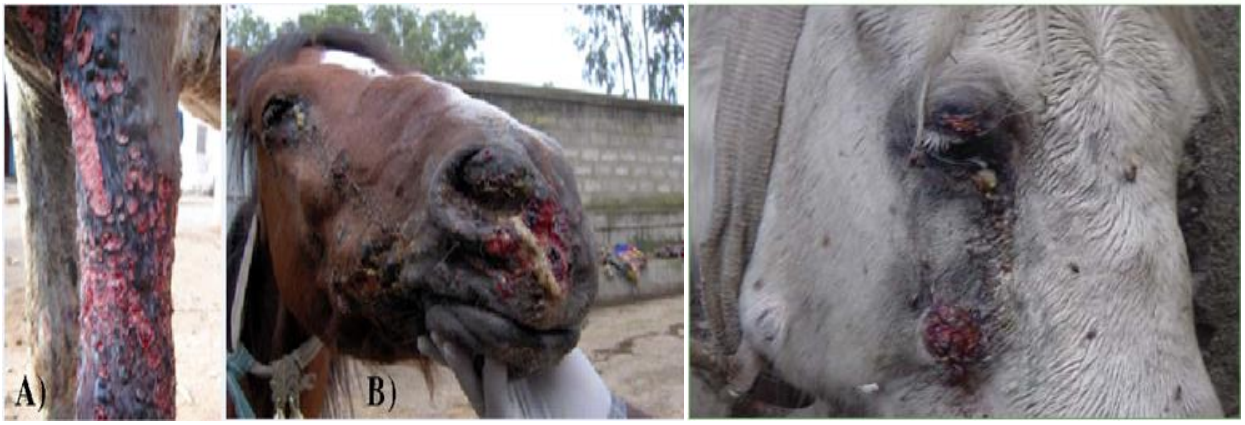

Figure1.Typical lesions of Equine Histoplasmosis A= Cutaneous form, B = Respiratory form and C = Occular form (source: Tagesu, 2017; Scantlebury, 2009)

2.3. Epidemiology

Histoplasma capsulatum is found in humans and animals, including horses, cattle, sheep, dogs, cats, chickens, rats, skunks and opossum (Lowa state University, 2009). Histoplasmosis is the most prevalent mycosis in North America and more often reported in the areas surrounding the Mississippi and Ohio rivers. Exposure to dust or soil for prolonged periods of time as well as activities which disturb bird and bat guano increase the risk of infection and have also been considered indicators of localized out breaks. Approximately 80 % of the general population tested around the Mississippi and Ohio rivers showed hypersensitivity to *Histoplasma capsulatum*. The largest groups of affected individuals were young adults.

Clinical EL affects horses, mules and donkeys (Scantlebury and Reed, 2009).The disease is endemic in some countries of West, North, North east Africa, East Africa and Asia including India, Pakistan and Japan. Humid and hot climates favour the occurrence and spread of the disease. The incidence of Equine Histoplasmosis become high in densely populated areas where large numbers of animal's population were collected together such as seen in military situations or congregation for cart horse transportation or racing. Mortality is low not exceeding 10-15% (Tagesu, 2017). Equine Histoplasmosis is commonly reported in horses in Ethiopia, especially in cart horses, affecting about 18.8% of them in warm, humid areas with elevation of 1500 to 2300

metres above sea level (Ameni, 2006). Prevalence as high as 26.2% was also recorded (Ameni and Siyoum, 2002). The detail of the situation of EL in Ethiopian equines is presented in section 2.7.

2.4. Transmission and Risk factors

Since the major portal entry of HCF to tissues is skin wounds, the occurrence of lesion is required for establishment of infections. Hence horses that are prone to lesion become directly or indirectly the destination of HCF through traumatized skin (Singh, 1966).Biting flies transmit the agent mechanically, inhalation of HCF and contact with fungal spore directly or indirectly with fomites, grooming material or beddings will allow the entry of the agent through skin abrasions. Sexual transmission is also reported (Al-Ani, 1999).Wounds from harnessing materials, tick bite and confinement of cart horses at place of work are found to be a major predisposing factor (Asfaw *et al.,* 2012). Sharing of harnessing and other materials among cart horse owners is the main method of spread of HCF in cart horses. The tradition of cauterisation and laceration to create painful skin wounds to hasten the rate work in carthorses also increase chance transmission.

2.5. Diagnostic Options

The major diagnostic methods for EL are clinical examination of the lesions, microscopic examination of the samples for yeast, serological tests and skin hypersensitivity test (OIE, 2018**).** In addition, molecular techniques have been developed and used for characterization of HCF during recent years. For example, PCR using the internal transcribed spacer (ITS) primers (ITS1 and ITS4) that are specific to the internal transcribed spacer region of rRNA genes of HCF has been developed. In addition, ITS1 and ITS2 regions of HCF have been used to sequence the gene for phylogenetic analysis and comparison with existing reference sequences in the data base (White *et al.,* 1990). Here after the conventional diagnostic methods commonly used for the diagnosis of EL are discussed.

2.5.1. Clinical examination

The clinical signs of EL in horses in endemic regions are often the basis of diagnosis; however confirmatory tests should be conducted (Tagesu, 2017). Spreading, suppurative, ulcerating pyogranulomatous lesion particularly in the neck, legs and chest can be used as an indication for cutaneous form of the disease. Ulcerating conjunctivitis and purulent nasal discharge, pyogranulomatous lesions around the nares supported with the above-mentioned signs can be considered indicators of ocular and pneumonic form of the disease, respectively. Clinical diagnosis of EL is however, limited to detection of clinical cases and it is not suitable for detection of early cases and carrier animals. Hence clinical diagnosis is not suitable for screening and isolation of positive animals so as to prevent spread of the fungus.

2.5.2. Direct identification of HCF

The agent of EL can be directly identified in samples collected from lesions based on the morphological features. When stained using Gram's staining HCF appear gram-positive, ovoid, pleomorphic ranging from 2–5 µM in diameter which can occur singly or in groups (Tagesu, 2017). It is frequently observed in smears prepared from purulent materials. The typical morphological appearance of the yeast form of HCF can be visualized using Giemsa or Periodic Acid–Schiff staining. Figures 2 a, b, c, d, e and f depict the morphological appearance of the yeasts of HCF in animals at different stages of EL. For morphological identification of HCF fine needle aspiration (FNA) biopsy collected form un-ruptured nodules and superficial lymph nodes are used. Smears prepared from these samples can be stained with modified Giemsa (May-Grunwald-Giemsa, Merck KGaA, Darmstandt, Germany) and examined microscopically (Rahmeto *et al.*, 2017).

Infection with HCF can also be revealed by the presence of multinucleated Langhans giant cells in histological sections stained with haematoxylin and eosin (basophilic mass surrounded by a halo), Periodic acid–Schiff stain and Gomori methenamine–silver stain. The appearance of the lesion is quite characteristic and consists of pyogranulomatous inflammation with fibroplasia (OIE, 2018). Electron microscopy has also been used for examination of skin biopsy samples immediately prefixed in phosphate buffered 2% glutaraldehyde solution at 4°C and post-fixed in

1% osmium tetroxide. Uranyl acetate and lead citrate stained sections has been used to study the fine internal structures of the organism, including the cell envelope, plasma membrane, cell wall, capsule and inner cell structures (Al-Ani, 1999).

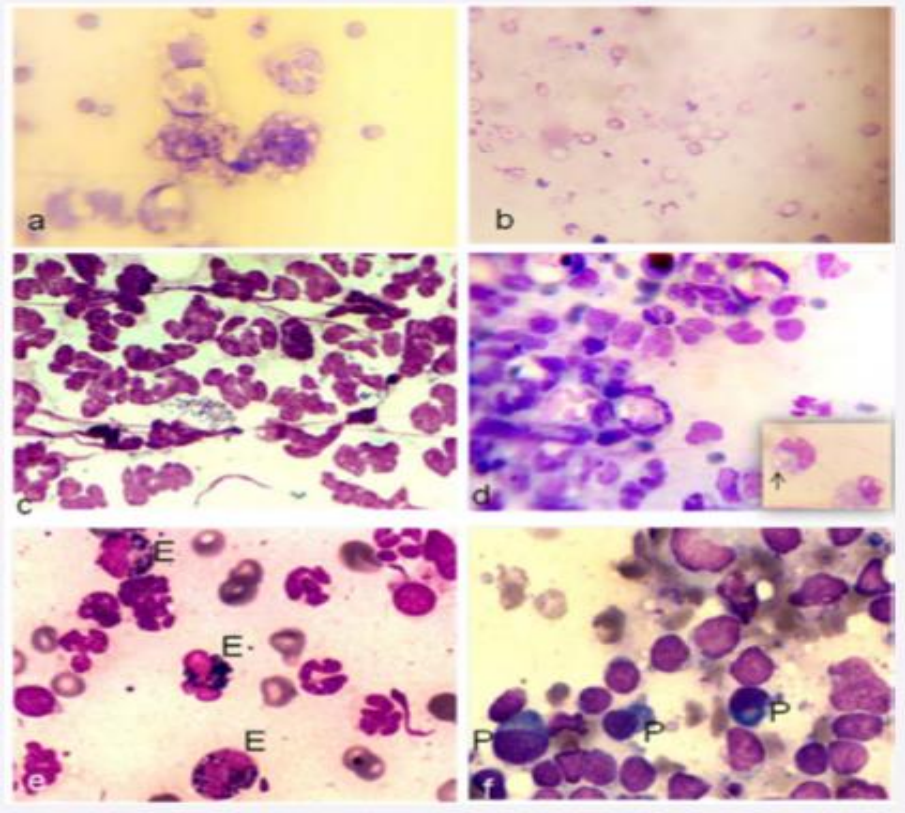

Figure 2. Fine needle aspiration smears stained with modified Giemsa : a. clinically active EL case with pyogranulomatous reactions with several HCF yeasts within white blood cells; b. several HCF yeasts outside white blood cells; c. lymphangitis with unidentified bacterial (smear made from nodule showing several degenerated neutrophils and pleomorphic bacteria), d. smear from apparently recovered case showing few number of yeasts in few macrophages and neutrophils e. lymphadenitis of immune stimulation from smear made from reactive lymph node, f. eosinophils and plasma cells (Rehameto *et al*., 2017).

2.5.3. Isolation of HCF

Culture of HCF from body fluids or tissues is considered the "gold standard" for confirmation of the diagnosis of EL but it is considered impractical since it is difficult and time-consuming to grow from body fluids and tissues. HCF has been cultured and isolated using Sabourauds Dextrose Agar (SDA) with 2.5% glycerol (Ameni *et al*., 2006). But it dies quickly in specimens, unless these are collected in antibiotic solutions and refrigerated and cultured promptly (Asfaw *et al*., 2012). HCF can also be isolated on other fungal media including mycobiotic agar, brain–heart infusion agar with 10% horse blood, and pleuropneumonia like organism (PPLO) media with 2% dextrose and 2.5% glycerol (pH 7.8). The HCF organisms grow slowly and colonies develop in approximately 2 to 8 weeks at 26°C. The colonies are dry, granular, wrinkled and grayish-white, becoming brown as they age (Lowa state University, 2009). The typical colony of HCF is presented in figure 3.

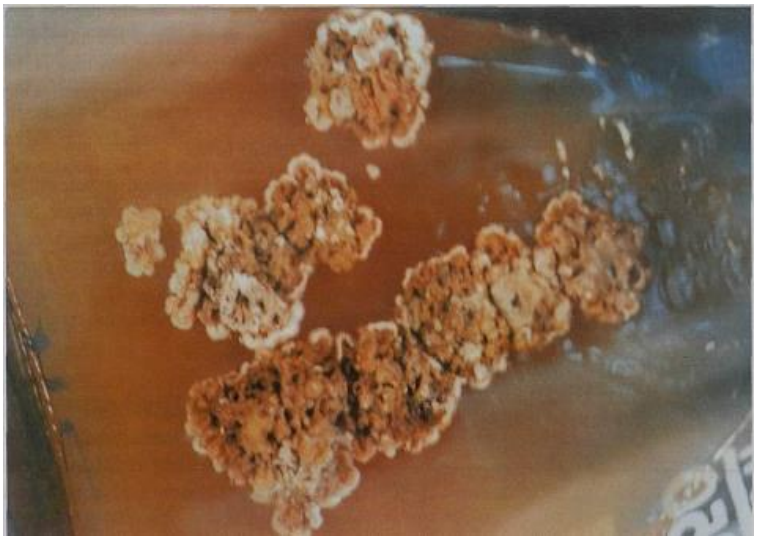

Figure 3. Colony morphology of HCF, grown on mycobiotic agar after 42 days of incubation at 26°C (Al-An, 1999).

2.5.4. Serological Tests

Serological tests such as direct and indirect fluorescent antibody tests, Enzyme Linked Immunosorbent Assay (ELISA) and Passive Heamaglutination tests have been used as diagnostic

tools for detection of antibodies in animals infected with HCF (OIE, 2018). However, these tests may not have a high degree of specificity for active disease search and may reflect past exposure and asymptomatic infection (Tagesu, 2017).Antibodies usually develop at or just after the onset of clinical signs. Tube agglutination and passive hemagglutination tests have been reported to identify increased titters in horses with EL, which can be used as a practical screening test. Indirect fluorescent antibody technique has been used as an aid to the diagnosis of infections by HCF in horses, using yeast phase cells of the organism as antigen. For example, in one study out of fifty equine sera from proven cases of HCF infections, forty-seven fluoresced brightly while fluorescent staining was absent in healthy controls and from horses suffering from commonly encountered ailments other than HCF infections (Fawi, 1965). An enzyme linked immuno sorbent assay was also evaluated for the detection of antibody in sera of equine naturally infected with HCF. In that study ten sera from naturally infected horses were tested and all were proved positive (Gabal and Mohammed, 1985).

2.5.5. Skin hypersensitivity test (Histofarcin test)

Skin test also known as histofarcin test has been applied for the detection of cell-mediated immunity (OIE, 2018) manifested as skin indurations as shown in figure 4. Histofarcin test could play significant role in detecting early infection and in the differential diagnosis of EL (Ameni, 2006).The duration of time for optimal skin indurations has been established to be with range of 24-48hours post inoculation of the antigen. Histofarcin test could be an innovative diagnostic option for EL. It is particularly useful in the border of endemic and disease-free regions where it is applied for early detection of infection and differential diagnosis from other diseases of equine like Glanders and Ulcerative lymphangitis (Ameni *et al*., 2006). It is very important diagnostic option for epidemiological investigation of EL.

The sensitivity and specificity reported (90.3% and 100%, respectively) (Ameni *et al*., 2006) of this test are found to be fairly high suggesting the potential application of the test for the diagnosis of the disease. Unlike other tests, histofarcin test enables to determine the immune status in individual animals or populations post vaccinations. However, the World Organization for Animal Health (OIE) reports in its 2018 module that this innovative procedure needs further

study and validation. Despite the advantages mentioned above, researchers reported certain limitations of the test such as result of false negatives and false positives and financial constraints for farther investigation of these results by slaughtering test animals for isolation of HCF from lymph nodes. The comparisons of the conventional tests, histofarcin test and molecular test for diagnosis of EL is given in Table 1.

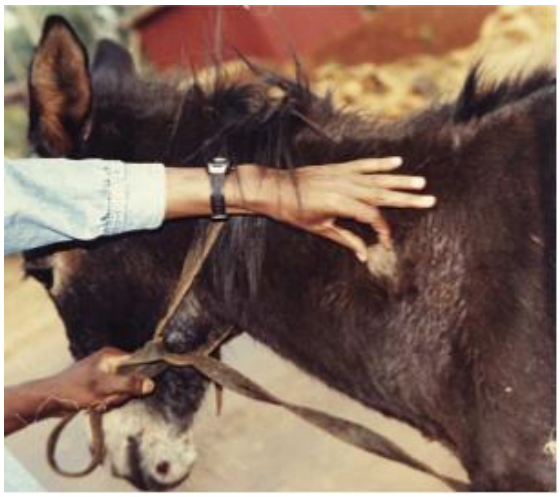

Figure 4. Local skin swelling post-intradermal injection of histofarcin on a clinically positive mule (Ameni et al., 2004)

Table 1. Comparative performance of diagnostic methods for diagnosis of EL (OIE, 2018)

Method	**Purpose**					
	Freedom from	movement control	For eradication	Clinical cases	Surveillance	Immune status
Antigen Identification						
direct smear	–	–	–	+	++	–
Culture	–	–	++	+++	+++	–
PCR	++	++	++	++	++	–
Detection of immune response						
FAT	n/a	n/a	++	++	++	
Indirect ELISA	n/a	n/a	++	++	++	–
Passive HA	n/a	n/a	++	++	++	–
Histofarcin test	n/a	–	++	++	n/a	++

Key: +++= recommended method, validated for the purpose shown; ++=suitable method but may need further validation; += may be used in some situation, but cost, reliability, or other factors severely limits its application; - =not appropriate for this purpose; n/a= purpose not applicable. PCR=polymerase chain reaction; FAT=Fluorescent antibody test, ELISA=Enzyme-linked immunosorbent assay; HA=haemagglutination.

2.5.6. Molecular diagnosis

The nested PCR were used as a diagnostic test for HCF cultures of clinical samples from infected horses. Two-stage nested amplification was done to manage the clinical diversity within the clinical samples. The diagnostic reliability of the nested PCR protocol was much superior to that of conventional microscopy or the sole reliance on clinical signs. In all cases, sequencing of the 514-bp amplicons demonstrated the presence of Histoplasma DNA, and this was further confirmed by sequencing of a large sample of clones (Scantlebury *et al.,* 2016).

2.6. Treatment Options

There is no complete satisfactory chemotherapy for EL. The recurrence of clinical signs has been reported in treated animals. The early case of the disease can be managed by surgical excision of the nodules on the affected parts and dressing of the wound with 4 per cent tincture of iodine. The infected animals can be treated with potassium iodide and sodium iodide by oral route and intravenous injection, respectively. Use of ketoconazole (orally) and Amphotericin B (intravenously) have shown good results in the treatment of horses affected with EL (Mahendra, 2012). In a research that evaluated Iodides, *Phytolaca dodecandra* – locally called “Endod”, and Penstrip for their therapeutic value on cases of EL, a combination of NaI and Penstrip had significantly higher therapeutic value as compared to other remedies (Table 2). The comparison on treatment response between the early stage and advanced stage of the disease showed that treatment during early stage provided better response (Birhanu *et al.,* 2007).

Table 2. Clinical response of EL to treatment options (Birhanu *et al*., 2007)

Treatment group	Early stage	Advanced stage	Total
	Number (%) cured	**Number (%) cured**	**Number (%) cured**
Endod +penstrip	3 (60)	3 (60)	6 (60)
Penstrip	2 (40)	0 (0)	2 (20)
Sodium iodide +penstrip	5 (100)	3 (60)	8 (80)
Potassium iodide	3 (60)	1 (20)	4 (40)
Endod	3 (60)	3 (60)	6 (60)
Potassium iodide + penstrip	3 (60)	2 (40)	5 (50)
Control	0 (0)	0 (0)	0 (0)

2.7. Significance of Equines

According to Food and Agricultural Organization (FAO) report there are about 58 million horses, 43 million donkeys and 10 million mules worldwide. Eight percent of the world equine population is found in Africa. Ethiopia is 1st in Africa and 8th in the world in equine population (FAO, 2011). The number of equines in the country is estimated to be about 2 million horses, 6.2 million donkeys and 0.38 million mules (Statistical Bulleitin, 2011). Equines especially horses have unforgettable role in the history of our world. They had been an engine for almost every society of the world and had played crucial roles in wars in different areas at different times. Hundreds of thousands of horses are died in the first and second world wars for instance. In addition to political roles played by equines during wars, particularly horses have unique places in many societies and have valuable social functions.

In Ethiopia for instance, horses provide various social roles and services during different festivals, celebrations and ceremonies as shown in figure 5 F and G. Equines are reared in different agro-ecological zones of Ethiopia for different purposes, including provision of draft power which can be used for ploughing and transportation (Guerin *et al*., 1992).

They are known to serve in transportation of different household materials, farm implements and agricultural goods from rural areas to nearby markets and then allow farmers to get market access (Wilson 1995), Supply of water in rural areas where water supply is limited (figure 5 D). Similarly, they have been used to transport farm inputs from commercial centres to the agricultural fields and contribute significantly to the crop production and food security (figure 5 E). Because of lack of infrastructure in the rural part of Ethiopia, most of the transportation activities are performed by the use of equines. As shown in figure 5 A cart horses are also used for transportation in the urban and peri-urban areas. Moreover, as the topography of the country is not convenient for modern transportation technologies, the major means of transportation both for goods and man are equines (Ameni and Siyoum, 2002). Figure 5 B shows the use of horses in transporting farm products such as maize to markets. Besides, in certain parts of the country equines are used for ploughing (figure 5 B). The services of equines can also be rented and provide income to the owners and the income obtained can be used to cover school fees for children, medical costs for the household members and to buy any other household items. That is equines in general do play important role in the livelihood of the society.


A
B


C
D


E
F

Figure 5. Equines on duty. A = horse providing public transport; B = horses being used for ploughing to prepare farm land; C = equines providing transport service for farm products; D = cart horse fetching water; E = equines transporting farms inputs; F and G = horses used for various social festivals

2.8. Epizootic Lymphangitis in Ethiopia

Epizootic lymphangitis is one of the major endemic equine diseases in Ethiopia causing considerable economic loss especially in carthorses. Prevalence as high as39% has been recorded in cart horses in central Ethiopia (Ameni and Siyoum, 2002). In another study conducted in 28 towns the average prevalence was reported to be 18.8% (Ameni, 2006). The highest prevalence (39%) was recorded at Mojo while the lowest (0.0%) was recorded at five towns, namely, Agaro, Bokoji, Debre Berhan, Dinsho, and Sagure. In similar studies the prevalence in 390 cart horses using clinical, direct smear and mycological examination as well as histofarcin test was found to be 24.9% (97/390) in Woliso (Rahel *et al*., 2012). Besides horses the prevalence of EL has been reported to be 32.84% (132/402) in cart mules (Demeke *et al.*, 2018). The prevalence in horses from different parts of the country is summarized in Table 3. Various factors are responsible for varying prevalence in the country.

Table 3. Prevalence of epizootic lymphangitis in horses and mules in 28 towns in Ethiopia (Ameni, 2006)

Town	Altitude in metres above sea level	Number of horses examined	Number positive	Prevalence (percent)	Average annual temperature (°C)	Average annual rainfall (mm)
Bati	1660	80	26	32.5	21	77
Kombolcha	1903	510	86	16.3	19.8	82.6
Harbu	1605	12	1	8.3	No data	75
Kemissie	1450	106	23	21.7	No data	77.1
Debre Birhan	2750	720	0	0	13.4	83.3
Debre Zeit	1900	690	204	30	19.8	90.5
Mojo	1870	520	212	39	19.8	88.3
Nazerath	1622	2100	420	21	21.4	93.9
Meki	1400	470	122	26.5	No data	70.3
Zeway	1640	240	67	28.2	21.4	71
Arsi Negale	1800	340	44	13.4	18.9	122.6
Shashemene	2080	3600	648	18	No data	50
Awasa	1750	4400	516	12.3	20.3	66
Jimma	1725	1600	600	37.5	19.9	107.1
Metu	1940	16	2	16.7	19.6	102.1
Woliso	2000	352	88	25	19.2	97.5
Nekemete	2080	72	8	11.1	19.3	156.4
Ejaji	1900	360	126	35	21.3	114.5
Agaro	2030	84	0	0	19.8	113.3
Bako	1650	100	10	10	No data	No data
Asela	2350	600	45	7.5	16	81.5
Sagure	2480	80	0	0	15.1	55
Bokoji	2800	80	0	0	No data	No data
Dodola	3000	400	40	10	No data	109.5
Adaba	2420	160	16	10	15.7	74.4
Dinsho	2450	10	0	0	No data	74.4
Robe	2400	1000	200	20	15.8	78.9
Goba	2700	500	75	15	No data	76.8
Total		19,082	3579	18.7559		

2.9. Control and Prevention

Outbreaks in non-endemic areas are probably best controlled by the slaughter of affected animals. The long incubation period of the disease, the high resistance of the causative agent and the presence of clinically healthy carriers made control of EL very difficult in endemic areas (Al-Ani, 1999). Quarantines and the euthanasia of infected animals are effective ways to control. Good hygiene and insect control will prevent the spread of the agent between animals. Cleaning and disinfecting of premises and harnessing materials and implements (grooming equipment and harnesses) thoroughly can help in preventing transmission of HCF. *Agent* can be inactivated by 1% sodium hypochlorite, glutaraldehyde, formaldehyde and phenolic disinfectants. This organism is also destroyed by moist heat at 121°C for at least 15 minutes (Lowa State University, 2009). Few studies have been conducted on vaccine trial, attenuated vaccine developed by exposure of the agent to high temperature administered and protection rate of 75.5% for 31 months has been reported (Al-Ani, 1999).

3. MATERIALS AND METHODS

3.1. Isolation and Identification of HCF

The study was conducted from October 2018 to May 2019 at Akililu Lemma Institute of Pathobiology, Addis Ababa University. Experimental study design and purposive sampling method was used. The HCF isolates used in this study were obtained from acute EL cases in cart horses found in different towns in central Ethiopia. Seven towns (Holota, Bishoftu, Mojo, Akaki, Gedo, Ijaji and Bako) were purposively selected based on the number of cart horses found and prevalence of EL reported from previous studies (Ameni, 2006). One hundred and fifty-seven cases with unruptured nodules were identified and areas around the nodules were thoroughly washed with water and soap. Using scalpel and blade washed area was shaved and disinfected with 70% alcohol. Then the content was aspirated using syringe and needle and transported to Addis Ababa University, Akililu Lemma Institute of Pathobiology laboratory.

Isolation of HCF from pus samples was conducted using Sabourauds dextrose agar enriched with 2.5% glycerol. Chloramphenicol (0.5g/L) was added to the media so as to avoid the growth of bacterial contaminants. Mycelial form of HCF was isolated after incubation at 26°C for about 8-12 weeks. The growth of dry, grey-white, granular wrinkled colonies on the agar suggested the growth of the mycelial form of HCF. The typical colonies were harvested, stained with Gram stain for confirmation of morphological features of HCF. Those isolates with typical colony appearance and morphological features of HCF were used for antigen preparation for skin test and evaluation of the effects of selected medicinal plants.

Figure 6. Cases of EL with ruptured and unruptured nodules from which samples were collected

3.2. Development and evaluation of histofarcin antigen for skin test

Colonies of HCF were aseptically collected from agar medium, and weighed. Roughly about 2mg/mL of the fungus was thoroughly ground using a porcelain mortar and pestle in saline solution. Further grinding was done using a motor grinder (Con-torque Power Unit, Eberbatch Corporation). The solution was then frozen-and-thawed. The frozen-and-thawed solution was sonicated using ultrasonic disintegrator at amplitude of 40° for 20 minutes. Thereafter, the solution was centrifuged at 4°C for 11 minutes and the supernatant was collected, its protein concentration determined by using bicinchoninic acid (BCA) protein assay as described by Pierce, BCT™ protein assay kit number 23225. The protein (histofarcin) antigen was used for histofarcin skin test with a final concentration of 0.2mg/mL. Zero point one (0.1mL) of the histofarcin antigen was injected into total of 30 horses, 10 that were infected with HCF,10 horses that were apparently healthy and 10 recovered cases. The injection was done intradermally using a needle having 27guage 1mL and 23guage 3mL into the middle of the neck. Before injection of the histofarcin, the skin was shaved and its thickness was measured using Bar Knight McLintock (McLK-P0005) calliper for each horse. The thickness of the skin was measured after 24, 48 and

72hours post inoculation. The test horses were considered positive if the thickness of the skin is greater than 4mm.

3.3. Preparation of Plant Extract

Three plants *C. longa*, *P. dodecandra* and *D. stramonium* (figure 7) were collected from field for screening of their effects against HCF. The plants were air dried and used for preparation of powder to be used for extraction of their contents**.** Methanol extracts were obtained by direct dipping of dry and powdered plant material at 1:10 ratio in 80% methanol for 48 hrs on an orbital shaker. The extract was first filtered by gauze on a sterile beaker and final filtration was done using Whatman filter paper (Number 1, diameter 6mm, Whatman ltd, England). The extraction process was repeated three times for exhaustive extraction. Methanol was then evaporated from the filtrate by using vacuum rotaryvaporator at 50°C and the extract allowed to remain in a micro oven at 40°c for one week for complete drying. The dried stock powder was kept in deep freeze for later use.

Serial two-fold dilutions of the extract were prepared using sterilized distilled water for *P. dodencandra* and *D. stratmonium* and Dimethylsulphoxide (DMSO, for *C. longa*) and finally concentrations of 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.312mg/mL, 0.156mg/mL and 0.07mg/mL were obtained. As a standard of treatment (positive control), dilution of ketoconazole was done at the concentrations of 0.8µg/mL, 0.4µg/mL,0.2 µg/mL, 0.1µg/mL, 0.05µg/mL, 0.025µg/mL and 0.0125µg/mL. Cultures of HCF with no treatment served as negative control.

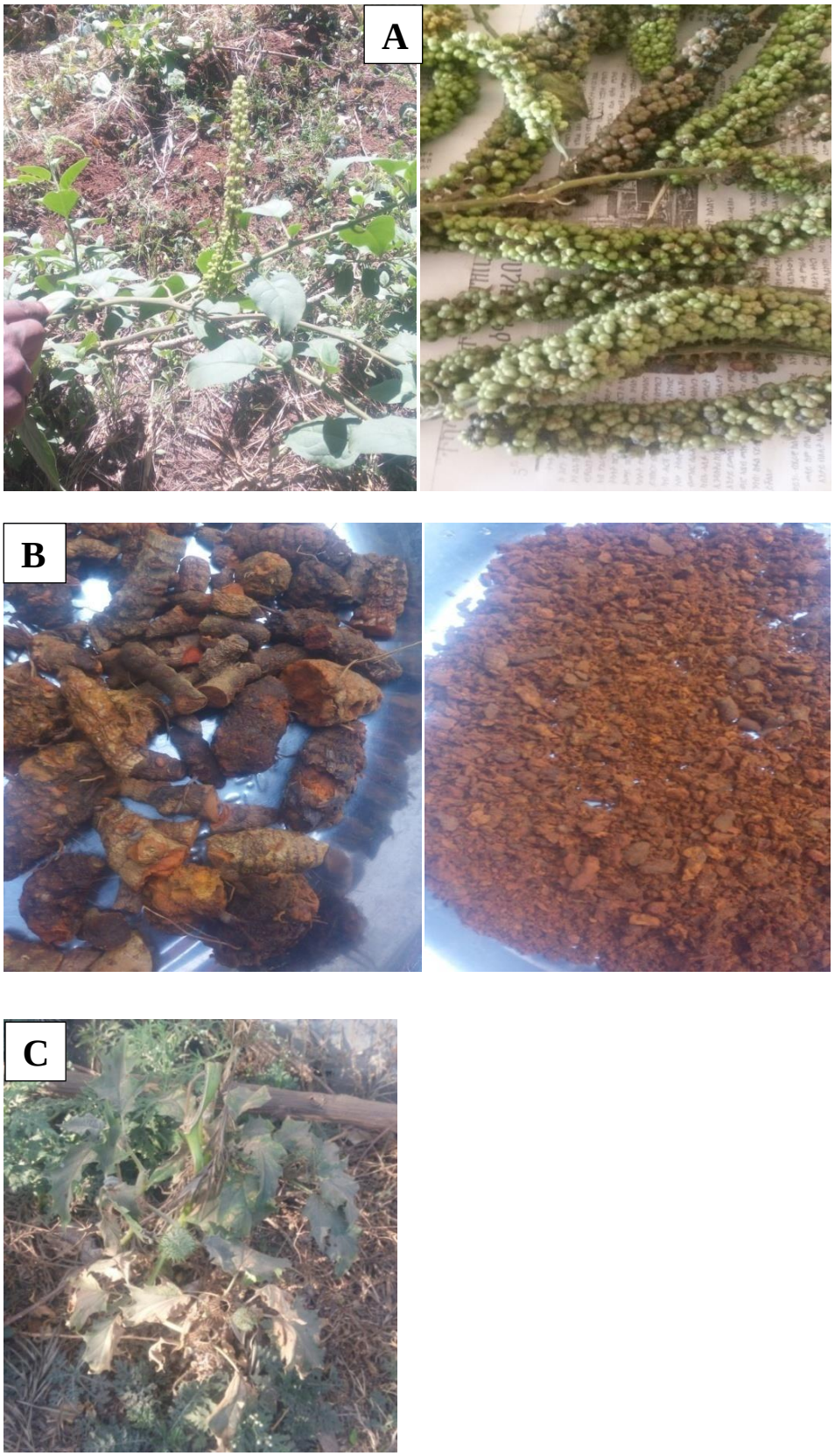

Figure 7. Plants used in this study A) *P. dodecandra* B) *C. longa* C) *D. stramonium*

3.4. Preparation and inoculation of media

Each concentration of the plant extract was added to sterilized universal bottles using micro pipette. Sabourauds dextrose agar media was prepared as described under section 3.1. Nine mL of the media was transferred to bottles containing each concentrations of plant extract and allowed to solidify after labelling. A well grown colony collected from sub-culture of a Mycelial form of the HCF was suspended in a saline water until turbidity matches 0.5 McFarland standard (which is equivalent to $0.4x10^4$– $5x10^4$cfu/mL) and uniformly streaked all over the agar surface by a sterile swab and incubated at 26°c. Positive control was prepared by culturing a fungal colony on SDA media with ketoconazol (standard) and negative control is prepared by culturing fungal colony on SDA free of any antifungal agent. The growth of HCF was monitored at least once per week.

3.5. Determination of MIC

Each concentration of the plant extracts was mixed with Sabourauds dextrose agar. The isolated HCF was cultured on each media containing the various concentrations of plant extract. The cultures were then incubated for 1 2 weeks. At the end of 12 weeks of incubation growth of HCF was inspected on each culture. The minimum concentration that inhibits the growth of HCF was considered the minimum inhibitory concentration (MIC) of *in vitro* growth of HCF.

DATA ANALYSIS AND RESULT INTERPRETATION

The diagnostic performance of histofarcin skin test antigen was assessed in reference to clinical sign, microscopy and history. The sensitivity and specificity as well as the positive and negative predictive values were determined from the result. As to the plant test media, the growth result was checked for each concentration and which plant inhibited the growth at which concentration was recorded and minimum inhibition concentration was determined form a concentration of last inhibition.

4. RESULTS

4.1. Isolation of HCF from field samples

A white grey to brown colony of HCF was grown after 12 weeks of incubation of needle aspirate samples on SDA enriched with 2.5% glycerol at 26°C. The typical colonies of HCF were identified as shown in figure 8 from samples collected from Holota (forty-five), Bishoftu (thirty four), Akaki (twenty) and Mojo (twelve) making a total of 111 isolates. In the entire positive samples gram positive, ovoid aggregate surrounded by a halo as depicted in figure 9 were observed. No case of EL was observed in Ijaji, Bako and Gedo districts during this study.

A)  B) 

Figure 8. Colonies of HCF after 12 weeks of incubation at 26°c. A) HCF Culture colony on SDA B) HCF sub-cultured colony on SDA

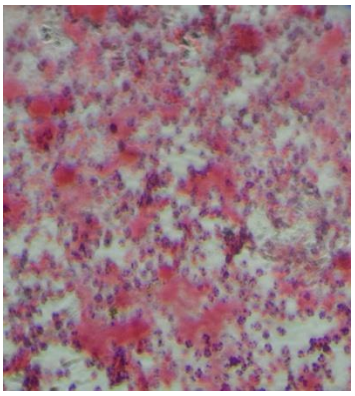

Figure 9. Typical morphological appearance of HCF in Gram stained smear

4.2. Preliminary evaluation of histofarcin skin test

A total of 30 (10 apparently healthy, 10 recovered and 10 clinically infected) horses were used to evaluate the diagnostic performance of histofarcin skin test. The result of this preliminary assessment is given in Table 4. Out of 10 clinically affected horses 8 of them tested positive using histofarcin test. That is, the sensitivity of the histofarcin skin test was 80% (95 % CI: 44.39 –97.47).Gram staining of pus collected from the unruptured nodules of two of the horses, which tested negative showed gram positive bacteria that suggest the occurrence of *Corynebacterium pseudotuberculosis,* which is the cause for ulcerative lymphangitis. Whereas out of the 10 healthy horses only 3 of them tested positive while 7 of them were negative yielding specificity of 70 % (95 % CI: 34.75 –93.32). Four (40 %) of the 10 recovered horses also tested positive. The positive and negative predictive values of the histofarcin skin test were found to be 53% and 86%, respectively.

Table 4. Result of histofarcin skin test in comparison with clinical status

		Infection status		
		Positive	**Negative**	**Total**
Histofarcin skin test	Positive	8	3	11
	Negative	2	7	9
	Total	10	10	20
	Sensitivity		**80 %**	
	Specificity		**70 %**	

4.3. *In vitro* Anti HCF effect of the plants

As shown in Table 5 five representative isolates were selected from each of three sites (Bishoftu, Holeta and Akaki) and four isolates selected from Mojo for evaluation of anti-HCF effects of the three plant species indicated above. The methanol extract of *C. longa* showed a good anti-HCF effect by inhibiting the growth of HCF at concentration ranging from 0.07mg/mL to 5mg/mL. That is, the minimum inhibitory concentration (MIC) of *C. longa* against HCF was 0.07mg/mL.The methanol extracts of *P. dodecandra* showed anti-HCF effects at concentrations

ranging from 0.156mg/mL to 5mg/mL, with MIC of 0.156mg/mL (Table 6). However, the methanol extracts of *D. stramonium* showed no effect on the inhibition of the growth of HCF. The positive control (ketoconazole) showed a MIC of 0.025µg/ml, while HCF grows on all the media with no antifungal agents (negative control).

Table 5. The number of isolates of HCF used to evaluate the *in vitro* effects of *P. dodecandra*, *C. longa* and *D. stramonium*

Site	**N^{0} cases**	**N^{0} isolates obtained**	**N^{0} evaluated**	**Response**
Bishoftu	62	34	5	effective
Holota	47	45	5	effective
Akaki	31	20	5	effective
Mojo	17	12	4	effective
Gedo	0	0	0	-
Ijaji	0	0	0	-
Bako	0	0	0	-

Table **6**. Results of the *in vitro* evaluation of methanol extracts of *C. longa*, *P. Dodecandra* and *D. stramonium* on HCF

C. longa		***P. dodecandra***		***D. stramonium***		**Ketoconazole**	
Conc.	**Growth**	**Conc.**	**Growth**	**Conc.**	**Growth**	**Conc.**	**Growth**
5mg/mL	X	5mg/mL	X	5mg/mL	+	0.8µg/mL	X
2.5mg/mL	X	2.5mg/mL	X	2.5mg/mL	+	0.4µg/mL	X
1.25mg/mL	X	1.25mg/mL	X	1.25mg/mL	+	0.2µg/mL	X
0.625mg/mL	X	0.625mg/mL	X	0.625mg/mL	+	0.1µg/mL	X
0.312mg/mL	X	0.312mg/mL	X	0.312mg/mL	+	0.05µg/mL	X
0.156mg/mL	X	0.156mg/mL**	X	0.156mg/mL	+	0.025µg/mL**	X
0.07mg/mL**	X	0.07mg/mL	+	0.07mg/mL	+	0.0125µg/mL	+
0.03mg/mL	+	-	-	-	-	-	-

Key: x= No growth observed
+= Growth observed

A)

5mg/ml 2.5mg/ml 1.25mg/ml 0.625mg/ml 0.312mg/ml 0.156mg/ml 0.07mg/ml 0.03mg/ml

B)

5mg/ml 2.5mg/ml 1.25mg/ml 0.625mg/ml 0.312mg/ml 0.156mg/ml 0.07mg/ml

C)

5mg/ml 2.5mg/ml 1.25mg/ml 0.625mg/ml 0.312mg/ml 0.156mg/ml 0.07mg/ml

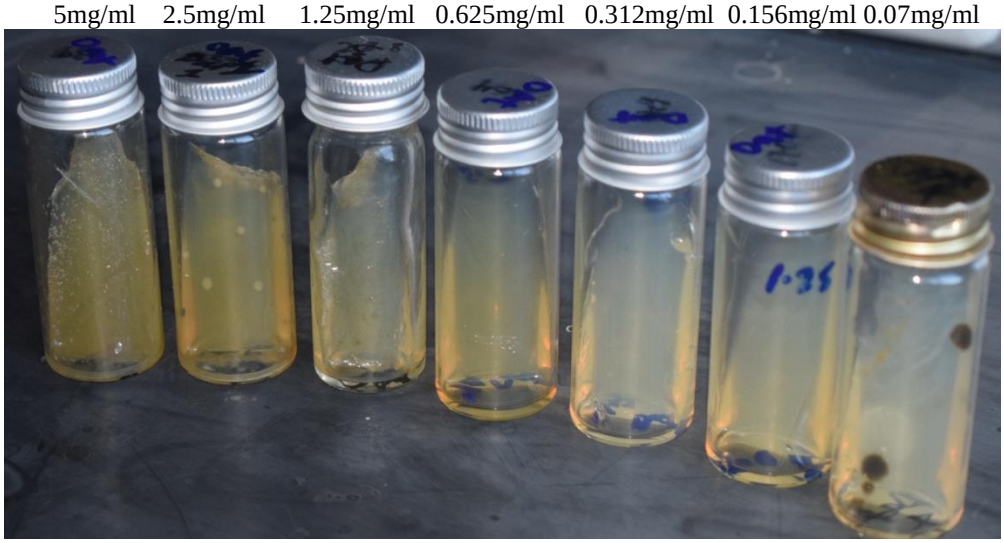

Figure 10. MIC of the plant extracts A) MIC of *C. longa* B) MIC of *P. dodecandra* C) MIC of *D. stramonium*

5. DISCUSSION

Horses especially cart horses play crucial roles in the livelihood of a number of Ethiopian rural and urban families. For some family they form the only means of survival and in several areas they provide the only means of affordable transportation service. They also provide draft power for ploughing and preparation of crop lands (Guerin *et al*., 1992). However, the optimum work output expected from horses and the benefits gained by the society are curtailed by diseases such as epizootic lymphangitis. It continued to incur considerable economic losses due to lack of appropriate pen side diagnostic methods and effective therapeutic and prophylactic options. Cheap and simple screening tests are needed for understanding of the epidemiology of the disease. Early detection of the disease and identification of the source of the disease is important for prevention and control. To this end we developed and evaluated histofarcin skin test and provided preliminary information on its utility. The availability of effective treatment is also crucial for management of the disease. There has been continual search for effective therapy for long periods of time. Hence, in this study we provided the preliminary *in vitro* effects of *P. dodecandra*, *C. longa* and *D. stramonium*.

The results of histofarcin test showed promising outcome with a good potential for detection of cases of EL. Despite the small sample size considered, the 80% sensitivity and 70 % specificity of the test is good performance. If the purification and assay for the level antigen in the preparation used is analytical grade the sensitivity and specificity of the test could have been better. In consent to our observation Ameni *et al*. (2006) reported a sensitivity of about 90%. That means, the histofarcin test is a promising alternative for screening horses particularly if they are not showing over signs of EL. The reactions of horses with different stages of EL shows the potential of this test for use at any stage during the development of the disease. One previous trial also suggested the possible use of histofarcin test in establishing differential diagnosis of diseases of equines confusing with EL such as ulcerative lymphangitis, glanders, strangles and sporotrichosis (Al-Ani, 1999). The observation of aggregates of rod shaped gram-positive bacteria in two horses having ulcerative lesions with negative histofarcin test result support this earlier observation.

A single study conducted previously on evaluation of histofarcin in Ethiopia reported 31 % false negative and 35% false positive results (Ameni*et al*., 2006). This is closer to our observation although we report a 20 % false negative and 30 % false positive results. The false negative results could be either due to other diseases producing similar lesions as EL since the classification of the status of the diseases was based on clinical signs or due to low level antibodies to produce visible reaction. The false positive results could be due the fact that horses were incubating the disease and sufficient immune response occurred to give reactions that increase the thickness of the skin although no visible lesions were produced. This needs further experimental investigation since knowledge of the status of the disease especially in subclinical and recovered cases is a basis for the effectiveness of a treatment and control of the distribution.

In vitro evaluation of the anti-HCF effects of *C. longa* and *P.dodecandra* provided promising results although *D. stramonium* did not have any effect. We provided preliminary evidence on the possible use of herbal remedies to search for alternative therapy for EL. This study also demonstrated that *C. longa* and *P. dodecandra* have anti-HCF effects at various concentrations. Both plants have comparable effects except that the MIC of *C. longa* was lower than that of *P.dodecandra*. Considering the lack of effective treatment for EL, this observation could be taken as remarkable. Although no study is available on the effect of *C. longa* on the growth of HCF isolated from equines, its powder has been considered best natural remedy for histoplasmosis in humans (Ciqionq *et al*., 2018). Previous studies have demonstrated the antimicrobial and antifungal effects of *C. longa* and its potential to treat gastrointestinal and respiratory diseases (Gupta *et al*., 2015; Ciqionq *et al*., 2018; Martins *et al*., 2009). It has also been shown that *C. longa* is immune booster (Chinampudur *et al*., 2013), which can considered as part of the management of EL. Al though analysis of its constituents and evaluation of its *in vivo* effects are awaiting, *C. longa* could be considered as a candidate for anti-HCF drug discovery.

This study also demonstrated the effect of *P. dodecandra* on the growth of HCF. A range of concentrations of methanol extracts of *P. dodecandra* was show to have anti-HCF effect with low MIC. In consent to our observation methanol extracts of *P. dodecandra* was shown to have good anti-HCF effects by earlier authors (Negesse *et al*., 2012). However, the current study demonstrated better effects of *P. dodecandra* with MIC (0.156g/mL), which is one-fourth of the

MIC (0.625) reported by Negesse and colleague. The previous study in contrary revealed that *P. dodecandra* inhibited growth of HCF at lower concentration than our observation when n-butane is used for extraction suggesting the potential impact of extraction methods. Phytochemical analysis carried out on *P. dodecandra* showed the occurrence of antifungal substances (Martins *et al*., 2009; Hadia *et al*., 2012; Negese *et al*., 2012) although their concentration vary based on ecological conditions. The results of this *in vitro* trial should be overlooked in attempts to search for effective treatment for EL.

Both *C. longa* and *P. dodecandra* have been shown to harbour metabolites such as alkaloids, flavonoids, phenols and saponins. These metabolites have long been known to have therapeutic properties against arrange of pathogens. The amount of these metabolites or the presence of other secondary metabolites, such as tannins, steroids and triterpenoids were reported to be abundant in *C. longa* (Gupta *et al.,* 2015) and might be responsible for the greater potential of this plant against HCF. The low MIC of *C. longa* against HCF observed in this study might be attributed to this property. These two plant species will play important role in the treatment and control of EL in the future. This could have important implication from economical perspectives in countries such Ethiopia where equines play crucial role in the livelihoods of millions of people.

In this study *D. stramonium* showed no effect on the growth inhibition of HCF. Even though there are no studies conducted on the effect of this plant on HCF, studies that investigated the effects of the ethanol extracts of *D. stramonium* revealed the occurrence inhibitory effect on fungi such as *Saccharomyces cerevisiae*, *Aspergillus fumigatus* and *Aspergillus niger*(Hadia*et al.,* 2012). In Ethiopia *D. stramonium* is used as an antifungal remedy to remove dandruff from human hair. The absence of anti-HCF effect observed in this study could be due to the extraction method used, biochemical properties of HCF or other factors affecting the concentration of metabolites.

Limitations of the Study

There were different difficulties/limitations faced in this study: first, the long incubation period needed to grow HCF resulted in overgrowth by fast growing fungal contaminants and limited our capacity for timely harvesting and processing of pure colonies of the fungus. Second, lack of

interest by the owners to be volunteer to conduct the skin test and to bring back their horses after two or three days to measure the skin thickness. Third, the lack of gold standard test to validate the histofarcin skin test limited our observation. Despite all these limitations we provided a preliminary finding that will play a significant role in treating and controlling EL in the future.

6. CONCLUSION AND RECOMMENDATIONS

This study revealed that histofarcin skin test showed a promising result to be used as a screening test with sensitivity of 80 % and specificity of 70 %. Two plant species: *C. longa* and *P. dodecandra* showed good inhibition effect on the growth of HCF. That is, these plants could be considered to be important alternatives for the treatment of EL in the future and should be considered important candidate in search for new and effective drug to be used against EL. hence, our observation has important implication for horse owners as EL is disease with a great welfare, health and economic impact. Based on these facts the following recommendations are suggested:

- Large scale assessment of the performance of histofarcin skin test is warranted
- Further investigation of the effects of *C. longa* and *P. dodecandra* including *in vivo* trials should be done
- Identification and purification of the active ingredients of the plants should be conducted

7. REFERENCES

Al-Ani F. (1999). Epizootic lymphangitis in horses: a review of the literature. Department of Veterinary Clinical Science, Faculty of Veterinary Medicine, *Jordan University of Science and Technology*. **18(3):**691-699

Ameni G. (2006). Epidemiology of Equine Histoplasmosis (Epizootic lymphangitis), in cart horses in Ethiopia. *Vet J*.**172(1)**:160-5.

Ameni G. and Siyoum F. (2002). Study on Histoplasmosis (epizootic lymphangitis) in cart-horses in Ethiopia. *J Vet Sci*.**3(2):**135-40.

Ameni G., Woldu T., Asrat H. (2006). Histofarcin test for the diagnosis of epizootic lymphangitis in Ethiopia: Development, optimization and development in the field. *Vet J* **171**: 358–362.

Asfaw R., Ameni G. and Mahandera P. (2012). Prevalence of Epizootic lymphangitis in Cart Horses in Southwest Shewa of Oromia Region, Ethiopia. *Int J Lives Res*.

Birhanu H., Ameni G. & Girmay M. (2007) Equine histoplasmosis: Treatment trial in cart horses in Central Ethiopia. *Journal of Tropical Animal Health and Production* **40(6):**407-11.

Ciqionq C. (2018). Antifungal activity, main active components and mechanism of *Curcuma longa* extract against *Fusarium graminearum. Plos on* **13(3)**

Demeke M., Rahmeto A. and Berhanu M. (2018). Prevalence of Epizootic Lymphangitis and Bodily Distribution of Lesions in Cart-Mules in Bahir Dar Town, Northwest Ethiopia. *J Vet Sci Technol* **9**:1.

FAO (2011)

Fawi T. (1965). Fluorescent Antibody Test for the Serodiagnosis Of Histoplasma Farciminosum Infections In Equidae. Ministry of Animal Resources, Khartoum, Sudan.

Gabal M., Khalifa K. (1983). Study on the immune response and serological diagnosis of equine histoplasmosis (epizootic lymphangitis). *Zentralbl. Veterinarmed.* **30,** 317–321.

Gabal, M. and Mohammed, K. (1985) Use of enzyme-linked immunosorbent assay for the diagnosis of equine *Histoplasmosis farciminosi*(epizootic lymphangitis).**91**: 35.https://doi.org/10.1007/BF00437284

Guerin C., Abebe S., Touati F. (1992). Epizootic lymphangitis in horses in Ethiopia. *Vet J*. Med Mycology; **2**:1-5.

Gupta A., Mahajan S., Sharma R. (2015). Evaluation of antimicrobial activity of *Curcuma longa* rhizome extract against Staphylococcus aureus. *Biotechnology Reports;***62**

Hadia G., Rubina N., Muhammad A., Shazia H., and Nabila Y. (2012). Antibacterial and antifungal activity of different extracts of Datura stramonium (branches and leaves sample). *Journal of Biotechnology and Pharmaceutical Research*; **3(9)**

Lowa state University College of Veterinary Medicine (2009). Epizootic Lymphangitis.

Mahendra P. (2012). Epizootic lymphangitis: A major fungal disease of equines in Ethiopia. Department of Microbiology, Immunology and Public Health, Faculty of Veterinary Medicine, Addis Ababa University, Ethiopia. *The Ethiopian Herald J.*

Martins C., da Silva D., Neres A., Magalhães T., Watanabe G., Modolo L., Sabino A., Fátima A., de Resende M. (2009). Curcumin as a promising antifungal of clinical interest, *Journal of Antimicrobial Chemotherapy*; **63(2).**

Negesse M., Eyasu M., Nigatu A., Gobena A. (2012). Evaluation of berries of *Phytolaccadodecandra*for growth inhibitionof*Histoplasma capsulatum var. farciminosum*and treatment of cases ofepizootic lymphangitis in Ethiopia. *Asian Pacific Journal of Tropical Biomediscine*

OIE (2018). Terrestrial Manual.

Rahel A., Mahendra P. And Gobena A. (2012). Prevalence of Epizootic Lymphangitis in Cart Horses in Southwest Shewa of Oromia Region, Ethiopia. *Int. J. Livest. Res.*. **2(3):**146-15.

Rahmeto A., Berhanu M. and Daniel K. (2017). Cytopathology of superficial lymph nodes and distended lymphatic nodules of horses with special reference to epizootic lymphangitis in Hawassa city, Ethiopia. *Ann Clin Pathol.* **5(5):** 1123.

Scantlebury C. and Reed K. (2009). Epizootic Lymphangitis, Review Chapter in Peer reviewed textbook; ' Infectious Diseases of the Horse'. *EVJ.*

Scantlebury C., Pinchbeck G., Loughnane P., Aklilu N., Ashine T., Stringer A., Gordon L., Marshall M., Christley R. and McCarthy A. (2016). Development and Evaluation of a Molecular Diagnostic Method for Rapid Detection of *Histoplasma capsulatum var. farciminosum*, the Causative Agent of Epizootic Lymphangitis, in Equine Clinical Samples.*J Clin Microbiol.***54(12)**:2990-2999.

Singh T. Studies on Epizootic lymphangitis: Clinical cases and experimental transmission. *Indian J vet Sci*. **36**: 45-59.

Statistical Bulleitin. (2011). Federal Democratic Republic of Ethiopia, Central Statistical Agency. Report on livestock and livestock characteristics. Volume 2, Addis Ababa, Ethiopia.Tagesu A. (2017). Review on Equine Epizootic Lymphangitis and its Impact in Ethiopia. Jimma University, College of Agriculture and Veterinary Medicine, School of Veterinary Medicine, Ethiopia. *Journal of Veterinary Medicine and Research* **4(8)**.

White T., Bruns T., Lee S., Taylor J. (1990). Analysis of Phylogenetic Relationships by Amplification and Direct Sequencing of Ribosomal RNA Genes. PCR Protocols: A Guide to Methods and Applications. *New YorkAcademic Press.*

Wilson R. (1995). The tropical agriculturist, live stock production systems, distribution and importance of live stock in the tropics. London CTA, Macmillan; **5**.

8. ANNEXES

Annex 1. Procedure for Sample collection

1. Clinically confirmed positive horses were identified
2. Areas with unruptured nodules were detected
3. The nodule and its surrounding was thoroughly washed with soap and water and disinfected with 70% alcohol
4. With a great care not to burst the nodule it was shaved with surgical blade and content was gently aspirated with a vaccinating needle.

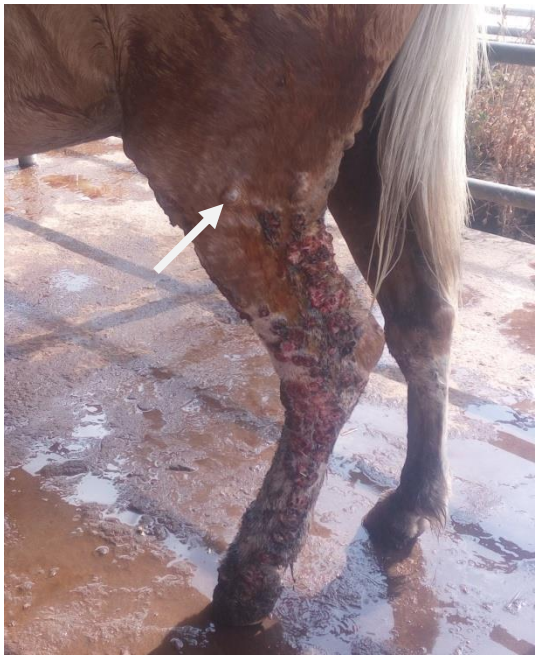

Figure- Washed, disinfected and shaved unruptured nodule ready for aspiration

Annex 2. Procedure for preparing a media and inoculation

1. A 65g powder Sabourauds dextrose agar was added to 1000ml of sterilized distilled water and boiled on a heater until the mixture dissolved completely and uniformly.
2. The media was then autoclaved at 121c for 15min and cooled.
3. After cooling 0.5g/l chloranphenicol and 2.5% glycerol was added and gently mixed
4. The agar was immediately poured to the bottles and kept in a slant position.
5. A drop of pus sample was put on the slant and striked with wire loop gently.

Annex 3. Procedure for preparing HCF antigen

1. Well developed colonies were removed from the media and weighed (2mg/ml)
2. By putting an eye glass to protect eyes colonies were ground manually in a normal saline by mortar and pestle further grinding was done by electric motor grinder
3. The solution was freez- thawed six times for further cracking of the particles.
4. Then by wearing an ear protector sonication of the solution with ultrasonic disintegrator at amplitude of 40° for 20 minutes in ice container.
5. finally, centrifuged at 4°c for 11min and supernatant was collected and kept at +4.

Annex 4. Procedure for determination of protein concentration of the antigen

1. **Preparation of Diluted Albumin (BSA) Standards**

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

2. **Preparation of the BCA™ Working Reagent (WR)**

2.1. Use the following formula to determine the total volume of WR required:

(# standards + # unknowns) x (# replicates) x (volusample) = total volume WR required

Note: 2.0 ml of the WR is required for each sample in the Test Tube Procedure, while only 200 µl of WR reagent is required for each sample in the Microplate Procedure.

2.2. Prepare WR by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B).

3. **Microplate Procedure (Sample to WR ratio = 1:8)**

3.1. Pipette 25 µl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 µg/ml).

Note: If sample size is limited, 10 µl of each unknown sample and standard can be used (sample to WR ratio = 1:20).

However, the working range of the assay in this case will be limited to 125-2,000 µg/ml.

3.2. Add 200 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.

3.3. Cover plate and incubate at 37°C for 30 minutes.

3.4. Cool plate to RT.

3.5. Measure the absorbance at or near 562 nm on a plate reader.

3. 6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.

3.7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

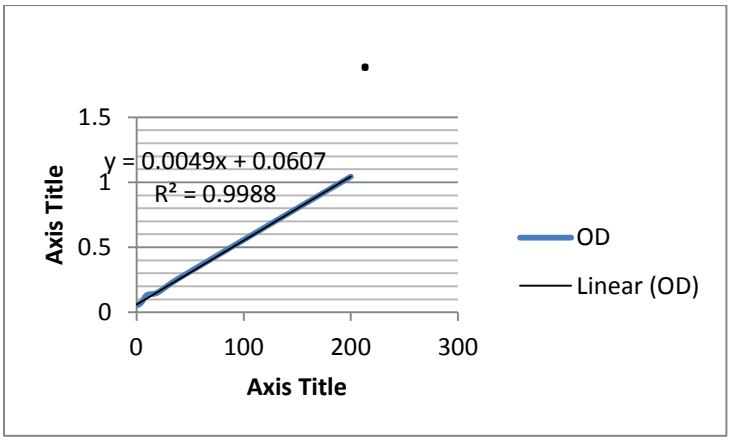

Figure: OD value and protein concentration graph for the histofarcin antigen

Annex 5. Procedure for plant extraction

1. Collected plants were transported to the laboratory and allowed to air dry.
2. Dried plant matter were ground by an electric motor grinder and dissolved in 80% methanol at 1:10 concentration.
3. The solution was allowed to stay on an orbital shaker for for 72hrs and it was filtered by gauze and watman filter paper, this process was repeated three times for exhaustive extraction of particles.
4. Finally methanol was evaporated by rotary vapor and solution was kept in an oven at 40°c for around a week to dry.

Figure: Grinding, filtering, evaporating and drying of the plant extract