Equine Epizootic Lymphangitis: Validation of Histofarcin Skin Test and an In Vitro Assessment of Growth Inhibition Effects of *Argemone mexicana* and *Zehneria scabra* on the Mycelial Phase of *Histoplasma capsulatum* var. *farciminosum*

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June, 2020

Bishoftu, Ethiopia

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COLLEGE OF VETERUNARY MEDICINE AND AGRICULTURE

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A Thesis submitted to Addis Ababa University

College of veterinary medicine in partial fulfillment of the requirements for the degree masters of Veterinary Medicine in Veterinary Epidemiology

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ACKNOWLEDGMENTS

First of all, I would like to thanks God who supports me in all activities throughout my life.

I am highly indebted to my advisors Dr. Teshale Sori, without his keen support, encouragement, insight, guidance and professional expertise the completion of this thesis would have been impossible. I would also like to thank him for his friendly treatment and devotion of time during the work of this thesis for his inviolable comment, material supports and editing of my thesis.

My appreciation will also goes to Dr. Musse Girma for his unreserved collaboration during laboratory work and field trip for data collection.

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LIST OF ABREVIATIONS

AGID	Agar-Gel Immunodiffusion
CF	Compliment Fixation
CSA	Central Statistics Authority
CVMA	College of Veterinary Medicine and Agriculture
EARO	Ethiopian Agricultural Research Organization
EL	Epizootic Lymphangitis
ELISA	Enzyme-Linked Immunosorbent Assay
GMS	Gomori Methen-amine-Silver stain
Hcf	*Histoplasma capsulatum* variety *farciminosum*
Hcc	*Histoplasma capsulatum* variety *capsulatum*
HE	Hematoxylin and Eosin stain
ID	Immunodiffusion
IP	Incubation Period
PAS	Periodic Acid Schiff reaction stain
SPANA	Society for the Protection of Animals Abroad

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ABSTACT

Epizootic lymphangitis is a chronic disease of equids caused by *Histoplasma capsulatum* var. *farciminosum*, resulting in remarkable loss in cart horses in Ethiopia. Absence of cheap and effective treatment and diagnostic tests are among the factors contributing to the widespread occurrence of the disease. In this study, histofarcin skin test was evaluated on 30 infected horses (15 clinically cases 15 recovered) and 15 healthy horses. Each horse was injected with 0.1mL of the histofarcin skin test antigen intradermally in the middle of the 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) caliper. The sensitivity and specificity of histofarcin skin test was 66.7 % and 100 %, respectively. In Vitro growth inhibitory effects of leaves and stem of *A. mexicana* and leaves of *Z. scabra* was evaluated. For this isolates of *Histoplasma capsulatum* var *farciminosum* were identified from clinical cases of EL in carthorses from six sites (Bishoftu, Akaki, Dukam, Asella, Shashemene and Gindhiir). Two-fold serial dilutions of the methanol extracts of the plants were prepared and assessed. Hcf culture media without antifungal agent used as negative control and media with ketoconazole taken as positive control. The result showed that A. mexicana and Z. scabra had inhibited Hcf growth at concentration 10mg/ml up to 2.5mg/ml. 2.5mg/ml was the minimum inhibitory concentration of the two plants. In conclusion, this study demonstrated that histofarcin skin test is a promising diagnostic tool for screening of equine histoplasmosis. Besides, the methanol extracts of *A. mexicana* and *Z. scabra* showed good *in vitro* growth inhibitory activity against the mycelial phase of *Histoplasma capsulatum* var *farciminosum*.

Keywords: Epizootic lymphangitis; Equines; Ethiopia; *H. capsulatum* var. *farsiminosum*; Medicinal Plants, Histofarcin Skin Test

1. INTRODUCTION

Horses provide a multitude of services to the community in Ethiopia (Hadush and Ameni, 2008) including draught power for ploughing in some parts of the country and transport throughout the country. However, the service rendered by horses is marginal as a result of several technical and non-technical factors. Epizootic lymphangitis (EL) is one of the technical factors curtaining the services of horses, particularly carthorses (Ameni and Siyoum, 2002). It is a debilitating fungal disease of equids, caused by a dimorphic fungus *Histoplasma capsulatum* variety *farciminosum* (*Hcf*). The mycelial form of the fungus survives in the soil in hot and humid areas while the yeast form is found in the lesions of infected equids (Radostits *et al*., 2010). It is closely related to *Histoplasma capsulatum* variety *capsulatum* (*Hcc*), which is a causative agent of histoplasmosis in humans (Radostits *et al*., 2010). In equids, EL occurs in three different clinical forms including the cutaneous, ocular and respiratory forms (Ueda *et al*., 2003; Ameni, 2007).

It's endemic in north, east and north east Africa. Also found in Mediterranean sea, India, Pakistan, and japan in Asia (Selim et al., 1985; Gurein et al., 1992; OIE, 2000) with varying prevalence. A study conducted in Ethiopia on 19,082 carthorses from 28 towns showed widespread occurrence with an overall prevalence of 18.8% (Ameni, 2006a). Besides, it was reported in 309 cart-mules with a prevalence of 21% in western Ethiopia (Ameni and Terefe, 2004) being associated with tick infestation (Ameni and Terefe, 2004; Ameni, 2007). The disease is particularly important in carthorses because it incurs considerable economic loss due to (1) reduced working hours because of morbidity, (2) reduced working life because of mortality, and (3) loss of horses due to mortality (Ameni, 2006a). It is one of the major welfare issues in endemic areas. EL 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).

Besides its economic impact due to its chronic and debilitating nature, equids suffer from illness due to EL for several months before death (Seid *et al*., 2019). Thus, other control options such as the use of medicinal plants could be better alternative approach. In addition to lack of effective therapeutic

approach, the absence of easy, rapid and cheaper diagnostic method, which is needed for understanding of the epidemiology of the disease, has contributed to the widespread occurrence of EL (Seid *et al.*, 2019). Continuous efforts are needed to reduce the economic losses incurred by the disease. These include continued search for effective treatment through screening of various plants against *Hcf*. Previous studies revealed that few plants had promising growth inhibition effects on *Hcf*. There are several plants that have been shown to have anti-fungal and anti-microbial effects against human fungal agents and certain livestock fungi. These include: *Argemone mexicana* (*A. mexicana*), *Zehneria scabra* (*Z. scabra*) (Chekole *et al*, 2015). These plants are supposed to have similar effects on Hcf and their growth inhibitory activities are investigated in this thesis. *In vitro* assessment of their effect is prerequisite for understanding of their pharmacodynamics property. In addition, evaluation of histofarcin skin test provides crucial step in the understanding of the epidemiology of EL and implement control measures. In recent investigation Hawi *et al*., (2020, article in press) showed that it has a sensitivity of 80 % and specificity of 70 % under laboratory conditions. Evaluation of the diagnostic performance of this test, however, is not done in the field. Therefore, the objectives of this study were testing effect of Z. scabra and A. mexicana on mycelial development of Hcf culture media and evaluation of the diagnostic performance of histofarcin skin test antigen under field conditions.

2. LITERATURE REVIEW

2.1. *Histoplasma capsulatum* variety *farciminosum*

Hcf is a thermally found in two forms and switches from a mold like (filamentous) form in the natural habitat to a small budding yeast form in the animal hosts (Untereiner et al., 2017). The filamentous mold form is found in the environment and can be cultured at 26.8ºC 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 37.8ºC, it grows to a yeast form, which appears as small oval buds of 2-4 micrometer. Macronidia are 8-15 micrometer and are thick walled with tubercles or projections. The micronidia are 2-4 micrometer and are smooth walled (Lowa state University, 2009). Under experimental conditions, inhalation of 5 yeast cells resulted in 10 % mice developing infection. The organism was first demonstrated in pus by Rivolta in 1873 but was not successfully cultivated until 1896 when the first pure cultures were obtained by Tokishiga in Japan (Al-ani, 2016) . The yeast form of the organism appears in pus as a double-contoured oval or ovoid body, measuring 2.5- 3.5 µm by 3-4 µm. The saprophytic stage is mycelial and both forms can be cultivated if suitable media, temperature of incubation and carbon dioxide tension are provided (Al-ani, 2016). *Hcf* has been known by various names such as *Histoplasma farciminosum, Cryptococcus farciminosum, Zymonema farciminosum, Histoplasma capsulatum* (Stringer *et al*., 2016).

Difficult to differentiate antigenically Hcf and Hcc. Genus Histoplasma classified into three different verities Hcf, Hcc and Hcd which classically expressed by equids and pathology (Adenis *et al*., 2014). Three variants have been described based on host predilection, clinical presentation, geographic distribution, or serology: *variant duboisii* in human in Africa, *variant farciminosum* in horses, and *variant capsulatum* for the majority isolates affecting humans and dogs in USA (Heitman and Filler, 2006). Histoplasma species now nowadays categorized in to eight clades based on multi locus type of sequence isolated and their location source (Kasuga, 2003).

The organism grows slowly when the yeast phase is grown on media rich in protein and in an atmosphere enriched with $C0_2$ (Awad, 1960).

The most satisfactory culture media includes SDA with 2.5 percent glycerol, BHI agar with ten percent horse blood, PPLO, nutrient agar with two percent dextrose and mycobiotic agar (Tagesu, 2017). Growth on all media is very slow and appears after four to eight weeks of incubation At 26.8 degree centigrade (Tagesu, 2017). Mycelial form of colonies of Hcf looks yellow or brown to deepest brown, folded, sticky and flower like. In body tissues, the ability of *Hcf* to convert from the mycelial form to the yeast form appears to be dependent on temperature and nutrition as well as the strain (Al-ani, 2016). However, in vitro, transforming of mycelial to the yeast of Hcf can be achieved by incubating at 37.8°C. Total transforming to yeast form of Hcf is by four to five time's repeated serial transfer to fresh media by eight days interval (Tagesu, 2017).

The biochemical characteristics of the mycelial form include positive reactions to catalase and urease tests as well as the assimilation of ammonium sulphate as the sole source of nitrogen. No fermentation of carbohydrate sugars, liquefaction of gelatine or reduction of nitrate occurs (Hadush and Ameni, 2008). The organism is highly resistant to the effects of physical and chemical agents (Hadush and Ameni, 2008) and can survive for at least a month in the dust of stables or kraals (Miki, *et al*., 2002). Al-ani found the pathogen to be viable and virulent after desiccation in the laboratory for 25 months (Stringer *et al*., 2016). Hcf may survive for up to ten weeks in non-sterile water at 26.8°C (Al-ani, 2016). Different nutrients and compounds are required for the growth of *Histoplasma capsultum* in different stages of its development. The nutritional requirements for the mycelia stage are simple; the organism can grow in a 26.8°C with glucose as its sole source of carbon and ammonia as its source of nitrogen.

During the yeast phase, *H. capsulatum* requires more complex compounds and a higher temperature environment. For the yeast phase, it first needs sulfhydryl containing for initiation of yeast development and cysteine or cystine along with certain growth factors such as biotin, thiamine, or thionic acid in order to maintain the morphology *(Johnson et al*., 2003). Cysteine plays an important role in the morphogenesis of *H. capsulatum*. It allows the cell to perform respiration for both phases and during the transition. There are two terminal oxidase pathways for *H. capsulatum*: the cytochrome

system which is blocked by cyanide and antimycin; the other, an unidentified alternative oxidase that is specifically blocked by salicylhydroxamic (SHAM) (Bossche *et al*., 1993). Another nutrient that is important for the growth of *Histoplasma capsulatum* is Iron. Iron is essential in redox reaction related to its existence in reduced Fe2+ and oxidized Fe3+ states. For a pathogen to be successful, it must have counter mechanisms for acquiring iron in the host microenvironment which it exists due to the limited amount of free soluble iron in an animal. There are a few approaches for the iron acquisition of *H. capsulatum* that have been studied. These strategies are: siderophores, acidic pH, reductive activities, and receptors for host iron binding compounds. None of these approaches is unique because they can also be seen in other organisms; however, the coexistence of such plethora in a single microbe is distinctive (Bossche *et al*., 1993).

2.2. Epizootic lymphangitis

EL is transmissible fungal illness of equids causing remarkable production loss and chronic infection in affected ones (OIE, 2008). Most cases of EL are reported from horses (90%), and the remainder from mules and donkeys. EL can occur in camels, cattle and dogs (Al-ani, 2016). It has also been reported in humans. Cattle are more resistant than equids (Al-ani, 2016). EL seen by following lymphatic border and diseasing skin, lymphatic vessel, lymph nodes and adjacent skin(Al-ani, 2016). EL is member of equine disease which negative impact on life of owner as well as equids life (Seid *et al*., 2019). It spread within animal itself and to other equines with Hcf loaded discharge, contaminated utensil and insects like fly and thick.

The most common form of this disease is an ulcerative, suppurative, spreading dermatitis and lymphangitis; however, other forms including pneumonia or ulcerative conjunctivitis also occur. EL spreads mostly where large numbers of equines are collected together. Epizootic lymphangitis is not found in some Europe areas and is also endemic in North, East West Africa, middle and Far East.

2.3. Epidemiology

The prevalence of EL varies with locations and the age. Horses under six years of age are most susceptible (Al-ani, 2016). EL is commonly found in the tropics and subtropics. It is endemic in north, east and north-east Africa, central Africa and some parts of Asia, and also some countries bordering the Mediterranean sea, Italy, India, Pakistan and Japan characterized by humid and hot climates (Al-ani, 2016). Some doubt exists concerning the validity of the reported cases of epizootic lymphangitis in the USA. EL is endemic in Ethiopia with the prevalence of 18, 8%. Historically EL was more common, when large numbers of Equines were collected together for cavalry and other transportation needs. The three major outbreaks of epizootic lymphangitis during the 20th Century have been associated with the massing together of large numbers of horses due to military operations (Irinyi *et al*., 2015). The disease mainly affects horses, mules and donkeys, although infection may occur in camels and cattle (Al-ani, 2016). Mice and rabbits may be infected experimentally (Ameni, 2007 and Scantlebury et al., 2015). EL is a contagious disease which can cause illness to humans (Scantlebury *et al*., 2015).

2.3.1. Mode of transmission

EL transmits by contacts, by fly and thick, by inhalation.it also enters in to the body through traumatized skin. The disease spread by contact with object contaminated by Hcf fungus like grooming objects, utensils used for feed and watering, harness and wound covering materials (Maxie, 2015). Harness related wound the first line to predispose the equids the disease in cart horse in Ethiopia (Ameni *et al*., 2006).

Flies that feed on open wounds were incriminated as possible vectors by Saunders as early as 1944 (Ameni and Terefe, 2004) and later Plunkett confirmed this conclusion (Rappleye *et al*., 2007). The organism has been isolated from the alimentary tract of biting flies that had alighted on open lesions, and the disease has developed in horses 4.8 km from the nearest case (Tagesu, 2017). Experimentally, flies (Musca spp. and Stomoxys spp.) have been shown to be capable of transmitting the infection. Transmission of the disease can occur when fly sit on lesion mechanically Scantlebury et al., 2015). Records also exist for the transmission of the disease from stallions to mares during copulation (Rappleye *et al*., 2007). The possibility of experimental infection in which the IP is much longer in

horse inoculated with mycelial organisms than that of with the yeast form (Ameni, 2006). Experimentally mycelial as well as yeast produce disease when inoculated with Hcf. Ticks Ambyloma and Boophilus transmit the disease which can be predisposing factors in mules (Ameni *et al*., 200).

2.4. Pathogenesis and Clinical Forms of EL

2.4.1. Pathogenesis

After enters to the body via opened wound by spreading tissue under skin create granuloma and disseminates through the lymphatics to regional lymph nodes or, in severe cases, to other organs. Nodular lesions develop in the skin along the lymphatics and in the lymph nodes. These lesions eventually ulcerate and drain a thick, mucopurulent material containing yeast cells. Nodules occur wherever there is skin trauma (particularly under the harness and on the extremities). Horses that have a heavy systemic burden of fungi may succumb to pneumonia or failure of other affected organs (Radostits *et al.*, 2010). Eye forms of the disease occur when a fungus enter to the eyes by flies (Radostits *et al*., 2010). Eye form and respiratory form occurred from skin forms by scratching their bodies with teeth and lips (Ameni, 2007). The incubation period (IP) of the disease is from 3 weeks to 12 months(Al-ani, 2016). Causes low mortality not more than 10% to 15% and emaciation and making the animal weak (Seid *et al*., 2019). Loss of productivity is from in ability of animals for long periods as a result of stress from the wound.

The OIE classify it as a list B disease (OIE, 2008). In endemic geographical location wind happened seasonally make the horse to inhale the fungus through dust leads to pneumonia (Tagesu, 2017). *Hcf* is highly resistant to the effects of physical and chemical agents. It may survive for up to ten weeks in non-sterile water at 26.8°C (Tagesu, 2017).

Gross lesions are manifested by pyogranulomatous, purulent discharge of thickened superficial lymphatic vessels and enlargement and inflammation of regional lymph nodes. Regional lymph nodes are swollen, soft, and reddened and may contain purulent foci. Lymphatic vessels may be found distended with pus. Skin fused with the under lying tissue and will be thickened in necropsy finding.

While thickened skin is incised, it presents the lardaceous appearance of granulation tissue and it contains a number of small, yellow, purulent foci between which the lymphatics are dilated and filled with pus and serous fluid (Al-ani, 2016).

2.4.2. Clinical Forms of EL

There are four different forms of EL: asymptomatic, cutaneous, conjunctival/ocular and respiratory. The first one, asymptomatic, occurs in patients which present fibro-calcified skin lesions at previous sites of infection and which are positive to intradermal sensitivity or other serological tests (Al-ani, 2016). Cutaneous forms of EL occur in horses when the organism enters to the skin. Animal get the fungus by contact with the diseased one through open wound, contact with contaminated materials as well as contact with contaminated bedding. Limb extremities, chest/girth region, neck and head are usual site for fungal entry. The disease will start anywhere on the bodies. Ones the organisms enter to the bodies immunological response leads to development of nodules at that site.

As Al-ani described Hcf will stay in digestive tracts of flies for 20 days pass them to healthy equids. No growth takes place in the flies. As Ameni showed there is positive relation between thick infestation and the disease. Fly transmit the disease within one animal from one site to another. Horse transmit lesion when grooming (Scantlebury, 2009).

In the early stages, the skin between the lesions remains normal and mobile, except in areas of extensive ulceration. The skin covering the nodules and the subcutaneous tissues may become thickened, fibrous, indurated, and firmly fused to the underlying tissues (Ameni, 2007). In the early stages, the swollen nodes contain many small foci of softening, but later the foci coalesce and are heavily encapsulated and they may rupture to form ulcers (Ameni, 2007). Fusing of lymphatic system develop pus containing granuloma that have a thick, fibrous capsule (OIE, 2008). The forelimbs, neck, and head are common sites to observe the Nodular and chronic suppurating lesions, however they are also observed on different body parts including the scrotal regions as shown in Figure 1. As the disease get chronic nodule burst and leading to more contamination and bad odor will happen.

Figure 1: Cutaneous forms of EL in a carthorse in Ethiopia showing Granulomatous inflammation of the lymphatic vessels, the regional lymph nodes and the skin of the fore limbs.

The ocular form: - Transmitted by flies feeding on eye discharge. Animal with trauma on eye are at great risk. Close used to swipe eyes pass infection to healthy ones. The ocular form of the disease may begin quite frequently on the conjunctiva or nictitating membrane, producing at first a small papule and a serous conjunctival discharge (Al-ani, 2016). Serous discharge from the infected eyes of mule is observed (Ameni, 2007). The papules ulcerate to form flat, button like growths of granulation tissue, the eyelids become severely swollen, and the inflammation extends to the tissues of the forehead (Al-ani, 2016).

A caple of nodules having risen surrounding found on mucosa of nose, conjunctivae, also on cornea as shown in Figure 2. Several purulent foci may be apparent on cut section (Al-ani, 2016). Sometimes the infection spreads into the facial tissues, seen as small nodules. If cases go untreated, secondary infections and severe ocular disease can occur.

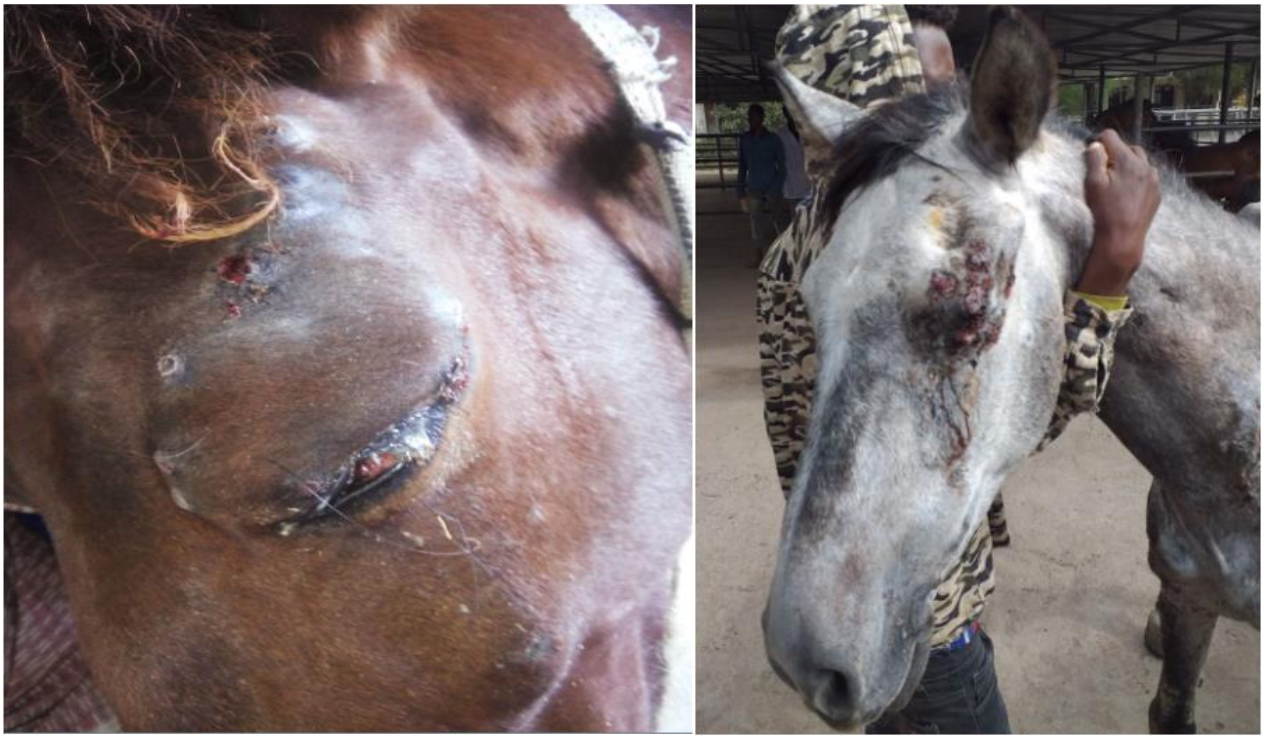


Figure 2: Ophthalmic form of EL: A button likes growth on the eyelid

The respiratory form: - Way of infection for respiratory form is not known. Awad guessed inhaling of spore may result pneumonia which may cause infection in lungs (Awad, 1960). Bruises on nares may cause for entrance fungus (Scantlebury, 2009).

The pulmonary lesions may be solid granulomatous areas or they may be liquefied with pus like contents (Al-ani, 2016). Nasal infection is usually accompanied by a mucopurulent discharge that may be bloodstained. On the nasal mucosa, the lesions begin as yellow flat papules or nodules on the nasal mucosa and these soon break down to form craterous granulating ulcers that bleed easily. The respiratory form of the disease is depicted in Figure 3. When ulcerative lesions are present on the nasal mucosa, there is suppurative regional lymphadenitis (Al-ani, 2016). Nodules and abscess could occur in the lungs in respiratory form of the disease (Ameni, 2007)

Figure 3: Pulmonary form of EL Source: (Seid *et al*., 2019)

2.6. Diagnosis

Diagnostic techniques include clinical testing, microscopy, serology, hypersensitivity testing (OIE, 2000). For confirmation culture is best. Aspirate pus from nodules after washing and dis infecting with 70% alchohol. We can see the organism by pathological staining (Scantlebury, 2009). In vivo test on mice also the other method of diagnosis (OIE, 2008). Ameni showed the gold standard for the validation of this test is Microscopic and mycological examinations of clinical lesions (Ameni, 2006b).

2.6.1. Serology

Serology when the there is no positive culture of *Hcf*, a presumptive diagnosis is usually made, based on the présence of antibodies in the sérum. Although several serological tests have been used for the diagnosis of epizootic lymphangitis, none of the tests are sufficiently sensitive or specific to confirm diagnosis.

The usefulness of the fluorescent antibody (FA) technique as a diagnostic tool for many infectious diseases has been firmly established. A number of investigators have explored the possibility of using the FA procedure for diagnosis of *H.c.var farciminosum* infection. The test is rapid and reliable, especially in cases where detection and isolation of the organism is unsuccessful. (Immunoglobulin G)

was used as a conjugate. The ELISA is simple and reliable for the diagnosis of the disease. In vivo testing by inoculating animal can be used for diagnosis facility.

Cell mediated immunity an accurate and reliable method of skin testing is the intradermal test. This consists of intradermal injection of 0.1 ml of soluble antigen prepared from *H.c. var. farciminosum*. An increase in the thickness of the skin of 8 mm to 20 mm, 24 h after injection of the antigen can be regarded as a positive result (Al-ani, 2016).

Tube agglutination and passive hem agglutination tests have been reported to identify increased titers in horses with epizootic lymphangitis, which can be used as a practical screening test. A serum agglutination titer of 1: 80 or higher is reported to be positive. Fluorescent antibody, AGID, and ELISA tests have also been described (Tagesu, 2017). ID and CF tests also have been reported as useful tests (Enbiyale *et al*., 2018).

2.6.2. *Direct Microscopy*

Giemsa, Diff-Quick, Gomorimethenamine silver good for staining of tissue (OIE, 2008). We use aspirated pus both for microscopic study and mycology. During sample collection unruptured nodules washed with soap, dis infected with 70% alchohol and sample aspirated with sterile needles. For pathological examination sample should be collected in 10% neutral buffered formalin. Sample sent to laboratory refrigerated and using ice box. For yeast form under microscope oil immersion at 100 magnifications is used.

2.6.3. Isolation

Isolation of *Hcf* done in slants of Sabourauds Dextrose Agar (SDA, Oxoid) it dies soon in specimens, unless collected in antibiotic solutions, refrigerated and cultured properly with chloramphenicol (0.5mg/ml) and 2.5% glycerol. For mycelia incubation temperature is 26.8 and for the yeast 37.8 degree Celsius (OIE). Grahams staining is done from suspected growth (Seid *et al*., 2019). By altering temperature from 26.8 to 37.8 we can change mycelial form to yeast form in the laboratory (Al-ani, 2016). White to grayish white, folded, raised cerebri form Colonies on SDA. Colonies when getting old

become brown. Yeast form is depicted as a gram positive with hole as a result of unstained capsule. Electron microscopy can also use.

2.6.4. Histofarcin (Skin) Test

It made by Soliman 1985. It is similar in principle with tuberculin skin test. where delayed type IV hypersensitivity showed first reaction exposure to organisms (Ameni et al., 2006). He reported s 90.3% and specificity 69% sensitivity. Ameni reported a sensitivity of the histofarcin test in the field to be 90.3%; however, specificity in endemic areas was 69%.

2.7. Treatment, Control and Eradication

Treatment of EL must to stop disease. Culling diseased used for control of disease and establishing hygiene measure is best. In recent years, the use of vaccines (i.e., a killed formalized vaccine, attenuated vaccine, and live vaccine) have been proposed as a strategy to eradicate the infection in endemic areas; the administration of the attenuate vaccine (i.e., vaccine developed by exposure of the causative agent to high temperatures) has resulted in a protection rate of 75.5% over >31 months (Ameni, 2006b). Intravenous dosing of iodide may be used particularly in endemic areas. The intravenous injection of 100 ml of sodium iodide of a 10% solution, repeated weekly for four weeks is recommended. Different antifungal drugs have also been used and successful treatment with amphotericin B has been reported (Radostits *et al*., 2010). Many treatment types have been tried, but it was without success. Amphotericin B injection best treatment. Even though Epizootic lymphangitis is highly prevalent and economically important in Ethiopia, the treatment options have not been employed because of the cost of the drugs and their absence in the market (Ameni, 2006b).

No effective treatment for disease. It's rarely responding to treatment mainly due to complication with different bacteria and its chronic nature. No effective medicine in Ethiopia is found for this disease. There is report with its response to medicine like amphotericin B. in other country. Traditional medicine practiced to treat some common hailments using plants available in Ethiopia. Natural products and their derivatives have been historically sources of therapeutic agents (Yirga, 2010). To treat epizootic lymphangitis in absence and inadequate of modern drugs, therefore the numerous plants are sources of antimicrobial agents, such as *combretum molle*, *xanthium strumarium*, which used to treat Epizootic lymphangitis in traditional way (Wondmnew, 2016). Control usually by avoiding the disease. To achieve this culling and keeping biosecurity is good (Scantlebury, 2009). Giving emphasis on grooming and good practice of harness material use is important.

This can be achieved by culling infected horse and donkeys and adopting strict biosecurity measures to prevent spread of the fungal agent(Scantlebury, 2009). Moreover emphasis should be taken to prevent spread by grooming or harness equipment usage methods.

2.8. Economic impact of Epizootic Lymphangitis

EL economically important disease in Ethiopia because life of Ethiopian farmer and cart driver dependent on horses and donkeys. Prevalence of EL in cart horse in Ethiopia is 19% and high loss of economy is lost (CDC, 2009). Healthy equines give much purpose and source of cash for those who their life rely on. Infections with EL decrease working ability which had direct effect up on livelihood of cart owner and their dependent family.

2.9. Status of Epizootic Lymphangitis in Ethiopia

2.9.1. Prevalence

The occurrence of EL has long been recognized in Ethiopia. Various studies carried revealed that it occurs in horses, mules and donkeys throughout the country with varying prevalence. The mean prevalence of EL in carthorses was reported to be 18.8% (Ameni, 2006a) and 14.7% (Muse, 2019). The prevalence of the disease is presented in Table 1 and Table 2 respectively.

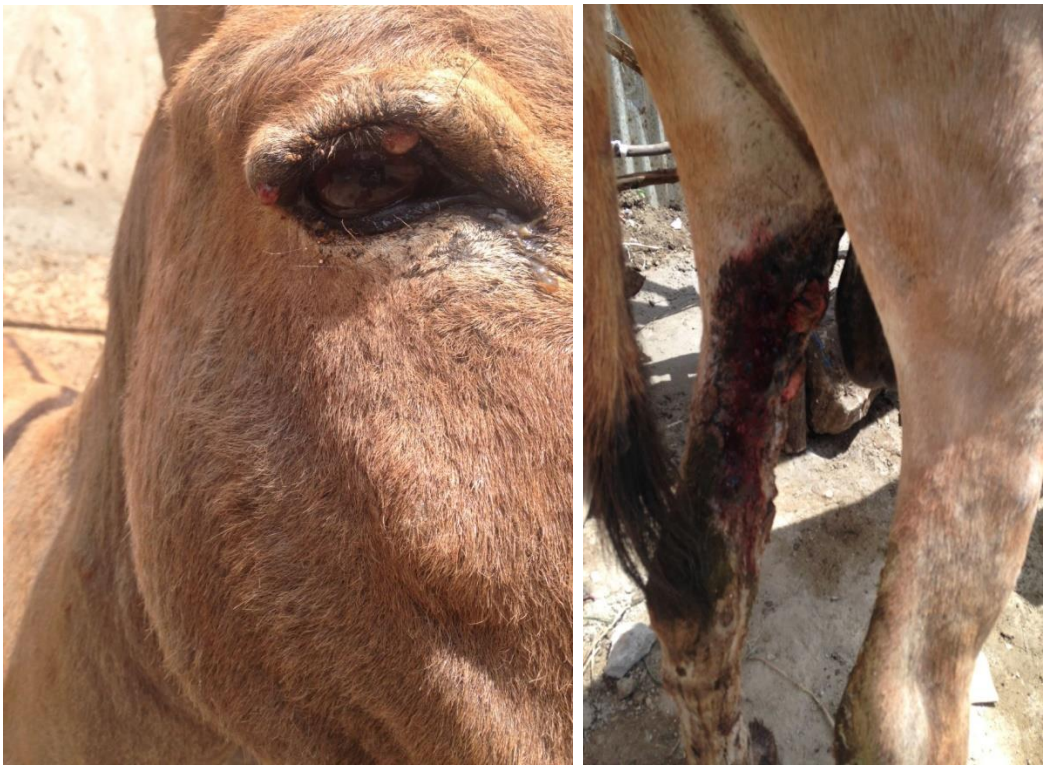


Figure 4: Cutaneous form of Epizootic Lymphangitis in a donkey observed in Gindhiir, Bale, Ethiopia.

Table 1: Prevalence of EL in carthorses in 28 towns of Ethiopia (Ameni, 2006a).

Town	**Altitude**	**N0 examined**	**N0 positive**	**Prevalence (percent)**	**Average annual temperature (°C) 21**	**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	78
Kemissie	1450	106	23	21.7	No data	77.1
D/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
A/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
Bakoji	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	74.7
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**	**18.79**		

Table 2: Prevalence of Epizootic Lymphangitis in carthorses of 16 towns of Amhara and Oromia Regional States of Ethiopia (Muse, 2019)

Town	Altitude in meters above sea leve	Number of horses examined	Number of positive horse	Prevalence in %	Average annual humidity in %	Average annual temperature in °C	Average annual rainfall in mm
Adama	1622	150	15	10	59.08	21.8	88
Bahirdar	1872	124	13	11	52.25	21.67	102
Bishoftu	1900	350	75	21	52.92	20.33	90
Debrebirhan	2750	234	0	0	49.75	16	No data
Dessie	2429	150	6	4	63.75	19	168.66
Dukem	2100	80	12	15	55.83	18.5	83.47
Ejaji	1900	300	5	2	66.25	20.5	150
Gondar	1973	384	33	9	49.75	20.92	110
Holeta	2391	307	91	30	57.67	17.67	93
Jimma	1718	302	60	20	67	20	100
Kemissie	1438	112	18	16	59.08	No data	80
Kombolcha	1811	251	84	34	63.75	21	80
Modjo	1870	200	8	4	48.42	21.5	90
Sebeta	2356	150	30	20	55.83	18.83	83.47
Shashaemene	2080	400	97	24	56.17	20.17	65
Woliso	2058	201	30	15	55.83	21	90
Total		**3,695**	**577**	**14.7**			

2.9.2. 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% 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. 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 3). 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). Besides, a recent investigation showed promising results on the use of medicinal plants against EL as shown in Table 4.

2.9.3. Diagnostic approach

Skin test also known as histofarcin test was developed applied for the detection of cell-mediated immunity manifested as skin indurations. The antigen used was developed at Akililu Lemma Institute of Pathobiology, Addis Ababa University and used for preliminary testing in horses and mules. It was found to be useful for early detection of infection and differential diagnosis from other diseases of equine like Glanders and ulcerative lymphangitis (Ameni *et al*., 2006). The same author reported a sensitivity and specificity of 90.3% and 100%, respectively. In recent investigation Hawi (2019; unpublished data) showed that skin test has similar sensitivity of 80 % (95 % CI: 44.39 – 97.47) and specificity of 70 % (95 % CI: 34.75 – 93.32). Molecular investigation showed that the isolates from the South American countries including Argentina, Brazil and Colombia are closely related to the Ethiopian isolates except the occurrence of nucleotide polymorphism at nine positions between the Ethiopian and the Cuban isolates figure 5. It represents a recent infection of Equidae by the South American Hcc following which the infected animals were transported to Africa and spread the infection among African Equidae via skin contact. Similarly, the high similarity among Ethiopian isolates and

that Indians as shown in figure 5 below could due the trading of sub-clinically infected equine species between African and Asian countries (Ameni et al., 2019; unpublished data).

Table 3: Clinical response of EL to various treatment options asses in Ethiopia (Birhanu *et al*., 2007)

Early stage Advanced stage	Total		
Treatment group	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)

Table 4**:** Results of the in vitro evaluation of methanol extracts of *C. longa, P. dodecandra* and *D. stramonium* on Hcf (Hawi, 2019, unpublished data)

C. longa		*P. dodecandra*		*D. stramonium*		Ketoconazol	
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/m	+	0.1µg/mL	X
0.312mg/mL	X	0.312mg/mL	X	0.312mg/m	+	0.05µg/m	X
0.156mg/mL	X	0.156mg/mL**	X	0.156mg/m	+	0.025µg/	X
0.07mg/mL**	X	0.07mg/mL	+	0.07mg/mL	+	0.0125µg/ mL	+

0.03mg/mL	+	-	-	-	-	-	-

Key: x= No growth observed; += Growth observed

Source: (Hawi, 2019, unpublished data)

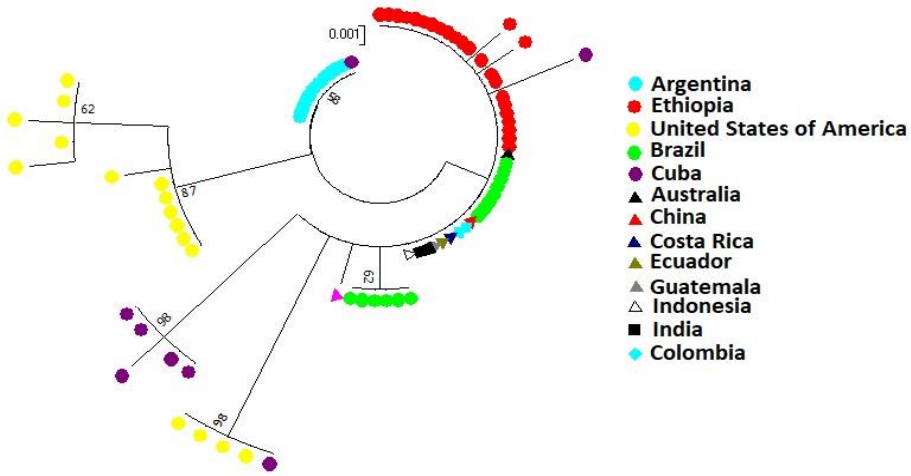


Figure 5: Phylogenetic tree constructed by maximum likelihood inference on the basis of the ITS1-5.8S-ITS2 sequences.

Sequences of Ethiopian isolates and those of other countries were included. As one can see from the phylogenetic tree there is geographical clustering; the Ethiopian isolates were related with Argentinean, Brazilian, Cuban, and Indian isolates. In contrast, isolates from North America were diverse and formed two distinct groups

3. MATERIALS AND METHODS

3.1. Isolation of *Histoplasma capsulatum* var *farciminosum*

The study was conducted from October 2019 to May 2020 at Akililu Lemma Institute of Pathobiology, Addis Ababa University. Samples of pus were collected from carthorses showing clinical signs of EL in different towns including Asella, Bishoftu, Shashemene, Akaki, Gindhiir and Dukam (Figure 6).

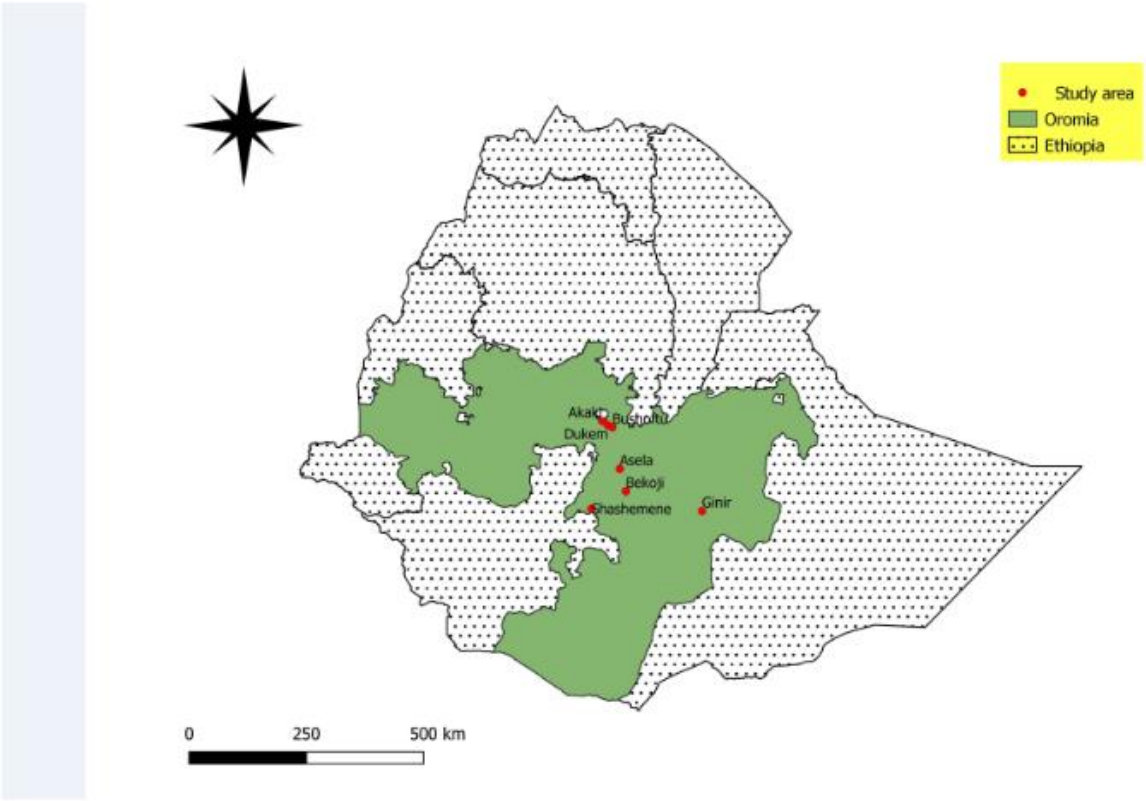
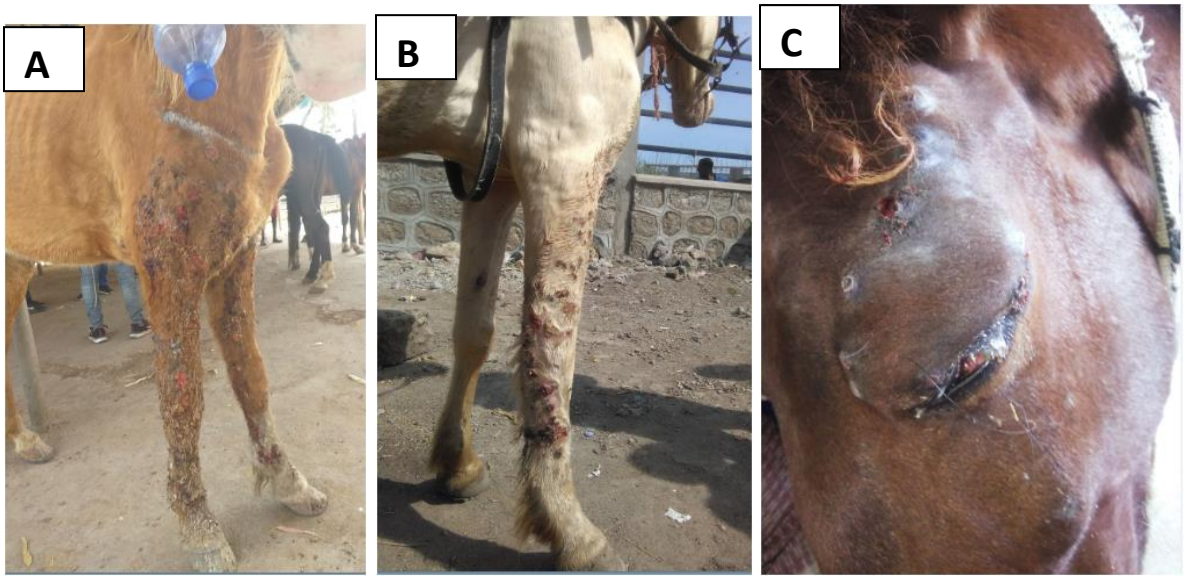


Figure 6: Map of Ethiopia showing field sites where pus samples were collected for isolation and identification of Hcf.

3.2. Sample Collection and Isolation of Hcf

One hundred one cases of EL with unruptured nodules were identified (Figure 7 A, B, C, D, E, F). The areas around the nodules were thoroughly washed with water and soap. It was shaved with scalpel

blade and disinfected with 70 % alcohol. The content of the nodule was aspirated using sterile syringe and needle. Each sample was transferred to universal bottle and labeled individually and transported to Addis Ababa University, Akililu Lemma Institute of Pathobiology using ice box for isolation of according to procedures described by OIE (2008). Isolation of the Mycelia form of Hcf was made on SDA and 2.5% glycerol, BHI with 10% horse blood and chloramphenicol (0.5g/liter) was poured to the media to avoid the growth of bacterial contaminants. The inoculated media were incubated at 26.8 °C for 6 to 8 weeks. The growth of the Mycelia was checked observed weekly. Whenever growth was evident the colonies were sub-cultured. The growth of the Mycelia form of *Hcf* was evident by the appearance of dry, grey to white/ yellowish, folded, raised, granular, convoluted, wrinkled, waxy and cauliflower-like colonies. The colonies fused to the medium and transform to brown on aging. Smears were made from typical colonies, stained with Gram stain for morphological characterization of Mycelia.



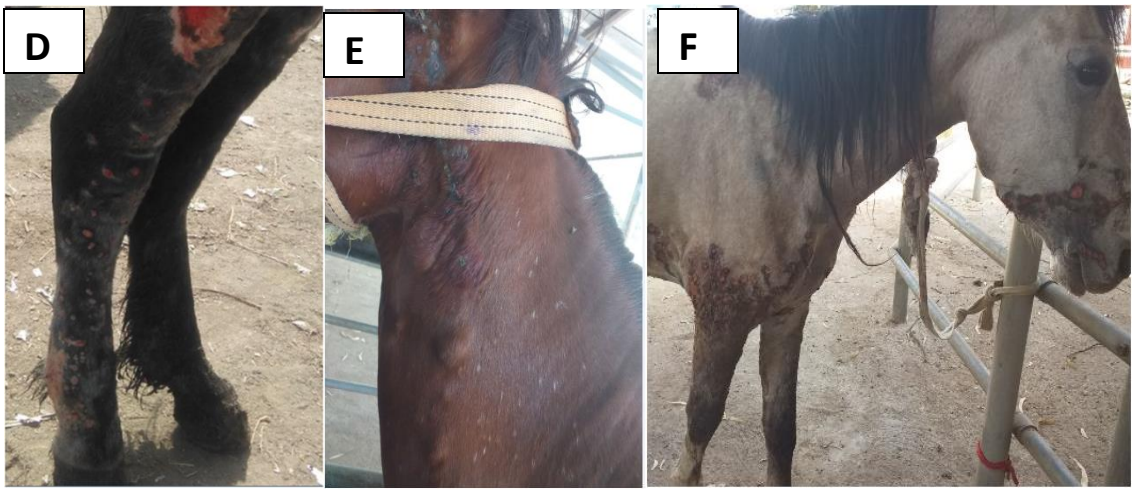


Figure 7: Clinical cases of EL with ruptured and unruptured nodules from which samples were collected

3.3. Molecular characterization of Hcf

3.2.1. Extraction of DNA

The modified SDS-CTAB-chloroform-isoamyl alcohol method was employed for the extraction of DNA (Umesha, 2016). Briefly 200 mg of mycelia was taken and transferred to 2 mL Eppendorf tube. Five hundred µL of SDS CTAB chloroform isoamyl alcohol (S-CCI) extraction buffer (250-mM Tris–HCl (pH 8.0), 20m MEDTA (pH8.0), 200M NaCl, 10% CTAB, 0.15 % SDS) was added and vortexed. It was centrifuged at 10,000 g for 10 minutes after boiling for 10 minutes at 50 ºC. The supernatant was aspirated carefully and mixed with one volume of chloroform: isoamyl alcohol (23:2) for 1 minute and centrifuged at 10,000 g for 5 minutes. The aqueous phase was collected and mixed with one volume of ice-cold isopropanol, and the tubes were turned upside down for 1 minute to precipitate the DNA. The tubes were again centrifuged at 10, 000 g for 2 minutes to recover the pellet and washed with 500 µL of absolute ethanol and then centrifuged at 10, 000 g for 1 minute. The pellet was air dried and the DNA was re suspended in 200 µL deionized or TE buffer. The DNA was either used for amplification or stored at -20 $^{\circ}$C until analysed.

3.3.2. Amplification and visualization

A nested PCR that amplify about 514 bp of DNA was employed as described by (Jiang *et al*., 2000; Scantlebury et al., 2016) using a pair of primers designated P3 and 2R8 supplied by Euro fins Genetics, Germany. The PCR was conducted in a reaction volume of 25 µL containing 1 µL DNA template (50 ng/µL), 12 µL Bio-Mix red (Bioline Reagents Limited, UK), 2 µL forward primer and 2 µL reverse primer (10 Pico mole each) and 8 µL H2O. The first round of the PCR was run using the P3 (forward primer (5′-CGGAAGGATCATTACCACGCCG-3′) and 2R8 (reverse primer (5′-CAGCGGGTATCCCTACCTGATC-3′) in the thermal cycler (Eppendorf Master cycler) with denaturation at 94 ^{o}C for 10 minutes followed by 35 cycles of the succeeding steps of denaturation at 94 ^{o}C for 1 minute, annealing at 49^{o}C for 1 minute and extension at 72 ^{o}C for 1 minute and a final extension period of 72 ^{o}C of 7 minutes. A one in ten (v/v) dilution the product from the previous reaction was added to new master mix and amplified using forward primer-F5 (5′-CTACCCGGCCACCCTTGTCTAC-3′) and reverse primer-2R5 (5′-CCTACCTGATCCAGTCAACC-3′). The reaction cycles for second phase PCR was the same as the previous phase, except that the annealing temperature was increased to 55degree Celsius for one minute. PCR products were visualized by gel electrophoresis using 2% agarose (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris-borate-EDTA buffer (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4]), stained with Ethidium bromide (3 µL per 100 mL agarose). The electrophoresis was conducted at 90 V for 50 minutes, with 5 in l of each PCR amplicons and loading dye in each well. The bands were visualized with a UV transilluminator.

3.4. Preparation of Histofarcin Antigen for Skin Test

Five pure colonies of *Hcf* were aseptically collected and ground thoroughly using a porcelain mortar (Con-torque Power Unit, Eberbatch Corporation) and pestle and suspended in 200 mL phosphate buffer saline solution. The solution was frozen and thawed five times. The frozen-and-thawed solution was sonicated using ultrasonic disintegrator at amplitude of 40° for 20 minutes.The solution was centrifuged at 1006 g at 4°C for 11 minutes and the supernatant was collected while the remaining mycelial elements were removed. Absence live fungus was checked by incubating an aliquot of the solution on Sabouraud's dextrose agar at 26.8 °C for 4 weeks. The concentration of histofarcin antigen was determined by using Bicinchoninic Acid (BCA) protein assay method described by Pierce

(Biotechnology, P. 2011). BCT^{TM} protein assay kit number 23225. The histofarcin- antigen was used for histofarcin skin test at a final concentration of 0.2mg/mL.

3.5. Evaluation of the Diagnostic Performance of the Histofarcin Skin Test

For the evaluation of the diagnostic performance of histofarcin skin test 0.1mL of the histofarcin antigen at final concentration of 0.2gm/mL was used in each horse. Injection of the antigen was done intradermally on the neck of each horse using a needle (27 G; 1mL). Before injection, the skin was shaved and its thickness was measured using Bar Knight Mc Lintock (McLK-P0005) calliper. The thickness of the skin was measured after 24, 48 and 72 hours after injection. Rising of skin thickness of greater than 4 mm was taken positive for infection with *Hcf* (OIE, 2008). The sensitivity and specificity of the histofarcin skin test was determined in 30 horses under field conditions. For estimation of sensitivity of the test 15 horses showing clinical signs of EL were inoculated in endemic areas. For determination of the specificity of the skin test 15 horses with no evidence of EL were inoculated with the antigen from areas where no cases of the disease has been reported. The sensitivity and specificity of the test was determined and reported.

3.6. Screening of Growth Inhibitory Effects of Plants

Fresh, *Argemone mexicana* locally called Dandaro (Amharinya); Medafe (Tigrinya), *Zehneria scabra* locally called Haregresa (Amharinya), were collected from Ada'a Bishoftu and Arsi Dhera Dodota. The plants used in this study are depicted in Figure 8. The plant materials were preserved following standard botanical procedures and submitted to Akililu Lemma Institute of Pathobiology for identification. Identification of the species of the plants was carried out by botanists based on the morphological characteristics, ecological preference and local nomenclatures. The leaves of the plants collected were washed thoroughly with clean water and air dried. The dried plant materials were garbled and grinded to yield fine powder for extraction. The powder was sieved using 250 µM wire mesh to get a fine material and then stored in zip lock bags at room temperature in dry place until use.

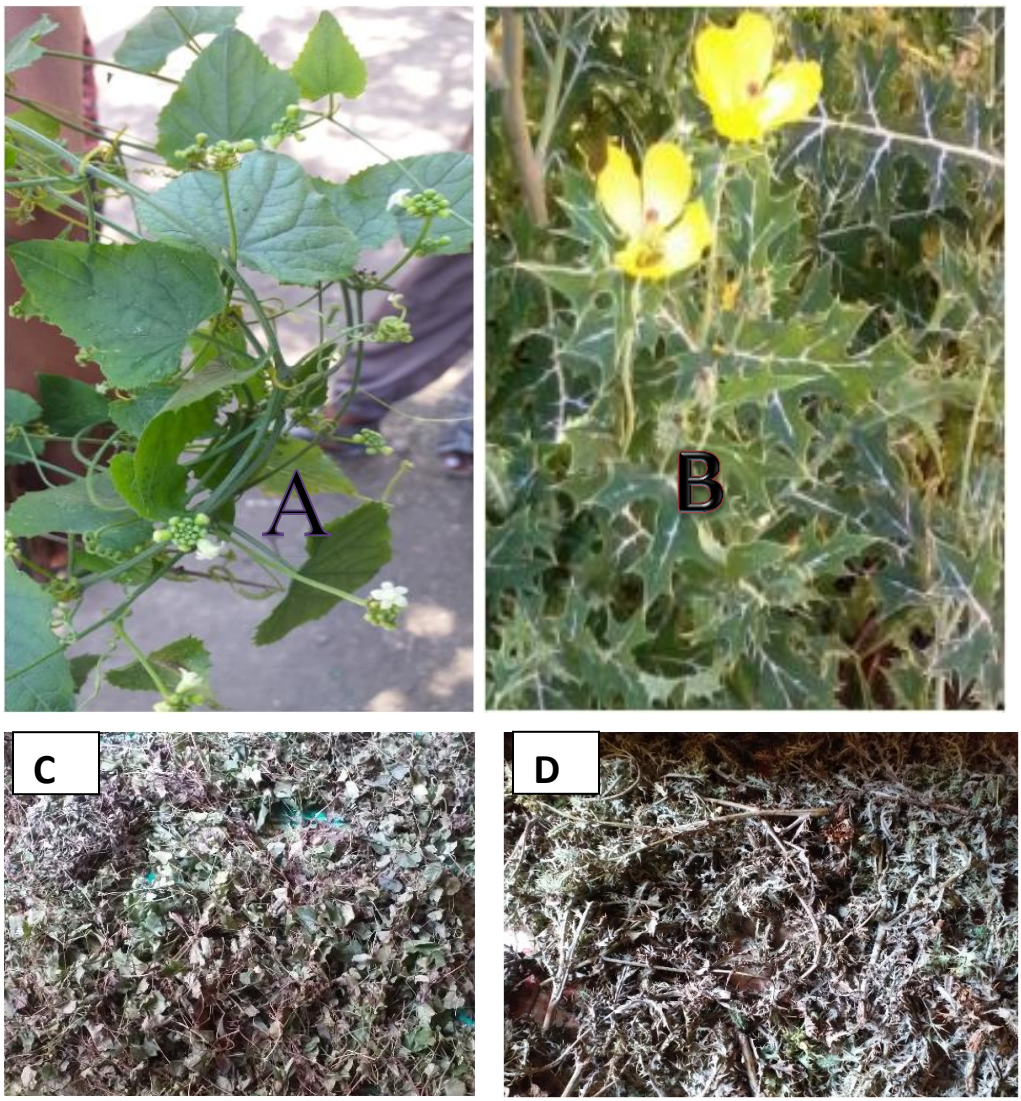


Figure 8: Pictorial demonstaration of fresh *Z. scabra* (A); fresh *A. mecicana* (B), dried *Z. scabra* (C) and dried *A. mecicana* (D)

About 133 grams of powder of each plant were mixed with 800 mL of 80% methanol (at ratio of 1:6 weight/volume) and shacked gently in Orbital shaker for 72 hours at 160 g. The extract was first filtered by using gauze into sterile beakers 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. The methanol was evaporated from the filtrate by using vacuum rotary vaporator at 50 ºC and kept in a micro oven at 40 ºC for one week. The dried stock powder was kept in deep freeze for later use.

The dried stocks powder were autoclave sterilized at 121 ◦C for 15 min at 15 psi to avoid contamination (Terrazas-hern *et al*, 2018). Stock solutions of selected medicinal plants methanol extract were prepared by dissolving 10000mg of selected medicinal plant extract in 10mL of pure dimethyl sulfoxide (DMSO). The solutions diluted with pure DMSO were then filtered with a filter paper (Whatman No. 1) and so the initial concentration of (1000mg/mL) were re diluted by transferring 1mL of the pre diluted sterile plant extract (solution diluted with pure DMSO) in 9mL of 5% DMSO (5mL DMSO added to 95mL Sterile distilled water) to found concentrations of 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, .6.5mg/ml, 3.12mg/ml, 1.56mg/ml and 0.7mg/ml. Then each 1mL concentration of the solution in 9mL of Sabourauds dextrose agar yields final concentration of 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.25mg/ml, o.312mg/ml, 0.156mg/ml and 0.07mg/mL of individual plant extracts. 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, 0.1 µg/mL, 0.05 µg/mL, 0.025 µg/mL and 0.0125 µg/mL. We used Hcf cultures without any treatment for negative control.

3.7. Preparation of the test fungus and inoculation

Each concentration of the plant extracts were added to sterilized universal bottles using micro pipette. Nine mL of Sabourauds dextrose agar media was transferred to bottles containing each concentrations of plant extract and allowed to solidify after labeling. Well grown colonies were scrapped by a sterile inoculating wire loop and transferred to a sterile saline (0.85%). Tween 80 (Sigma) was added in order to wet the molds and then mixed by shaking vigorously using Vortex mixer until turbidity matches 0.5 McFarland standard (which is equivalent to 0.4x104 – 5x104cfu/mL). The density of the test suspension was compared with that of the standard and it was adjusted by adding either more fungal colony or sterile saline and the prepared agar dilutions of the extract and controls were inoculated by dipping sterile swab into the test suspensions. Excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid suspension. Then, the swab was streaked all over the surface of the agar medium in radial pattern. Finally, the lids of the inoculated agar tubes were closed and incubated at 26.8 °C for 8 weeks with follow up (CLSI, 2008). Positive control was prepared by culturing a fungal colony on SDA media with ketoconazole (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.

4. RESULTS

4.1. Isolation of *Histoplasma capsulatum* var *farciminosum*

A white grey to brown, yellowish, granular, folded, raised, waxy and cauliflower-like wrinkled Hcf colonies were grown after 12 weeks of incubation of needle aspirate of pus samples at 26.8 °C on Sabouraud dextrose agar with 2.5% glycerol and BHI agar added with 10% horse blood. The colonies were attached to the medium and become brown on aging. Smears were made from typical colonies, stained with Gram stain for morphological characterization of mycelia. The typical mycelial colonies of Hcf were identified as shown in Figure 9.The results of Wright-Giemsa were presented in Figure 10.

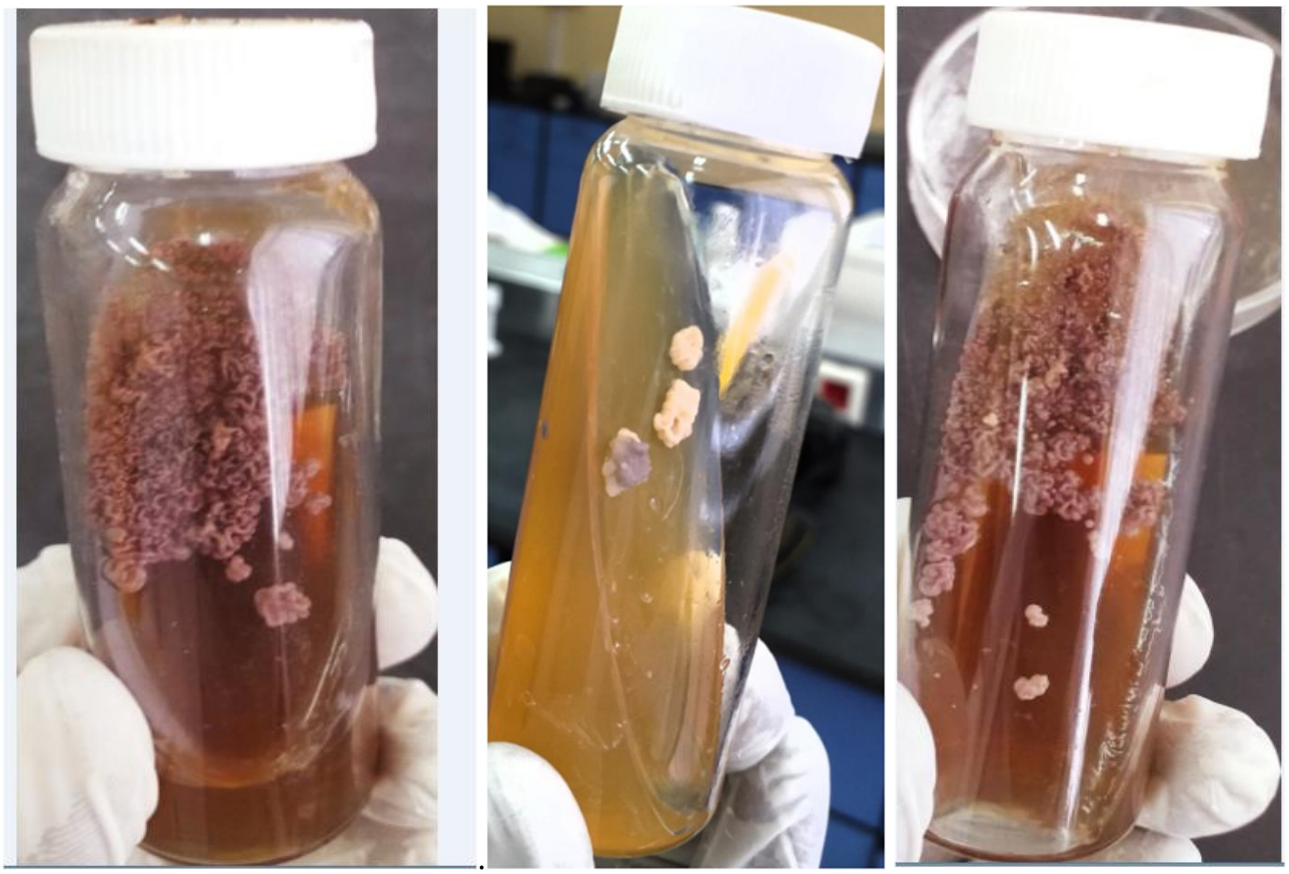


Figure 9: Typical colonies of the mycelial form of Hcf after 12 weeks of incubation at 26°c on SDA

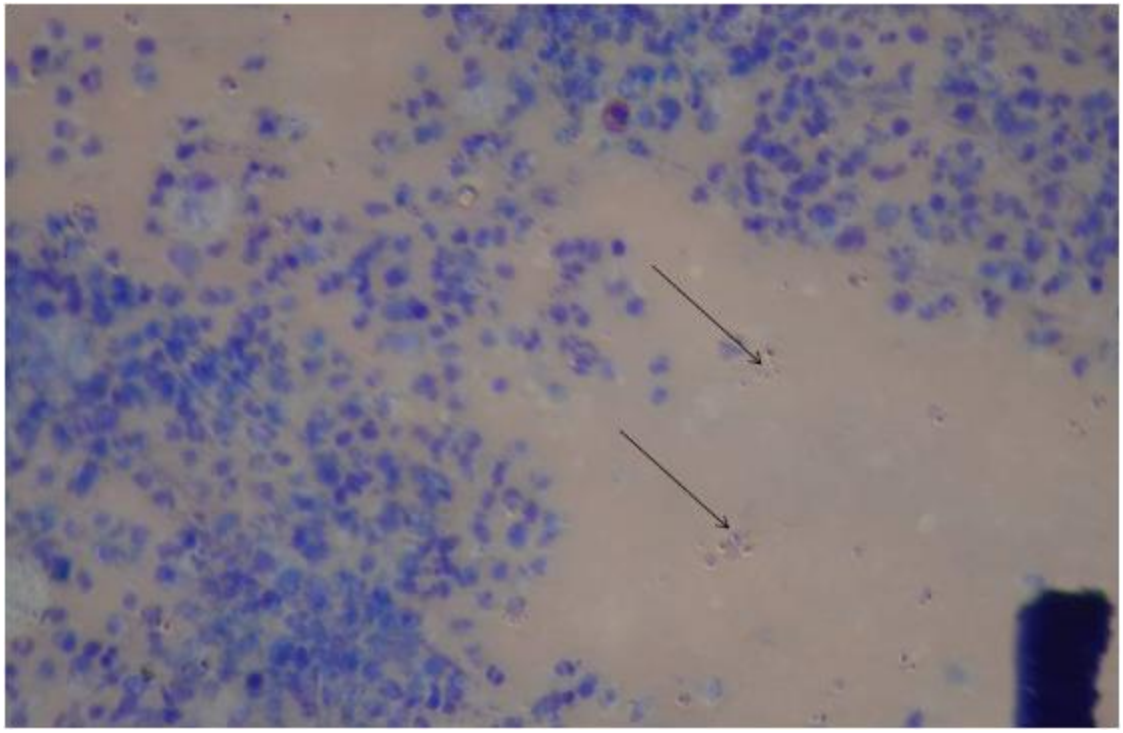


Figure 10: Microscopic appearance of Hcf in Wright-Giemsa stained smear made from a pus sample demonstrating tissue form of Hcf inside with abundant round to spherical yeast-like cells (100 xs).

4.2. Preliminary Evaluation of Histofarcin Skin Test

A total of 45 (15 apparently healthy, 15 recovered and 15 clinically infected) horses were used to evaluate the diagnostic performance of histofarcin skin test. Out of 30 infected horses (15 clinically affected and 15 recovered horses) 20 of them tested positive using histofarcin skin test antigen as showed in Figure 11. That is, the sensitivity of the histofarcin skin test was 66.67% (95 % CI: 47.19, 82.71). 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 15 healthy horses 15 of them were test negative yielding specificity of 100 % (95 % CI: 81.89, 100.0). 7 (47 %) of the 15 recovered horses also tested positive.

Figure 11: Result of histofarcin skin test antigen after 48 hours of intradermal injection

4.3. In vitro Anti Hcf effect of the plants

As shown in Table 6 five representative isolates were selected from Bishoftu, Asella and Akaki, four from Gindhiir and Dukam, and two from Shashemene for evaluation of anti-Hcf effects of the two plant species indicated above. The methanol extract of both *A. mexicana* and *Z. scabra* showed a good anti-Hcf effect by inhibiting the growth of Hcf at concentration 2.5mg/mL and 10mg/mL. 2.5mg/Ml was the minimum inhibitory concentration of A. mexicana and Z. scabra against Hcf. (Table7; Figure 12). 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: Result of histofarcin skin test in comparison with clinical status

		Infection Status		
		Positive	**Negative**	**Total**
Histofarcin				
skin test	Positive	20	0	20
	Negative	10	15	25
	Total	30	15	45
	Sensitivity		**66.67%**	
	Specificity		**100 %**	

Table 6: The number of isolates of Hcf used to evaluate the in vitro effects of *A. mexicana* and *Z. scabra*

Site	№ cases	№ isolates obtained	№ evaluated	Response
Bishoftu	55	49	5	effective
Asella	17	12	5	effective
Akaki	8	5	5	effective
Gindhiir	7	5	4	effective
Shashemene	4	3	2	effective
Dukam	10	7	4	effective
Overall	**101**	**81**	**25**	**effective**

Table 7: Results of the *in vitro* evaluation of the growth inhibitory effects of methanol extracts of *A. mexicana* and *Z. scabra* on Hcf

A. mexicana		*Z. scabra*		Ketoconazole	
Conc.	Growth	Conc.	Growth	Conc.	Growth
10mg/mL	X	5mg/mL	X	0.8µg/mL	X
5mg/mL	X	5mg/mL	X	0.4µg/mL	X
2.5mg/mL**	X	2.5mg/mL**	X	0.2µg/mL	X
1.25mg/mL	+	1.25mg/mL	+	0.1µg/mL	X
0.625mg/mL	+	0.625mg/mL	+	0.05µg/mL	X
0.312mg/mL	+	0.312mg/mL	+	0.025µg/mL**	X.
0.156mg/mL	+	0.156mg/mL	+	0.0125µg/mL	+

–

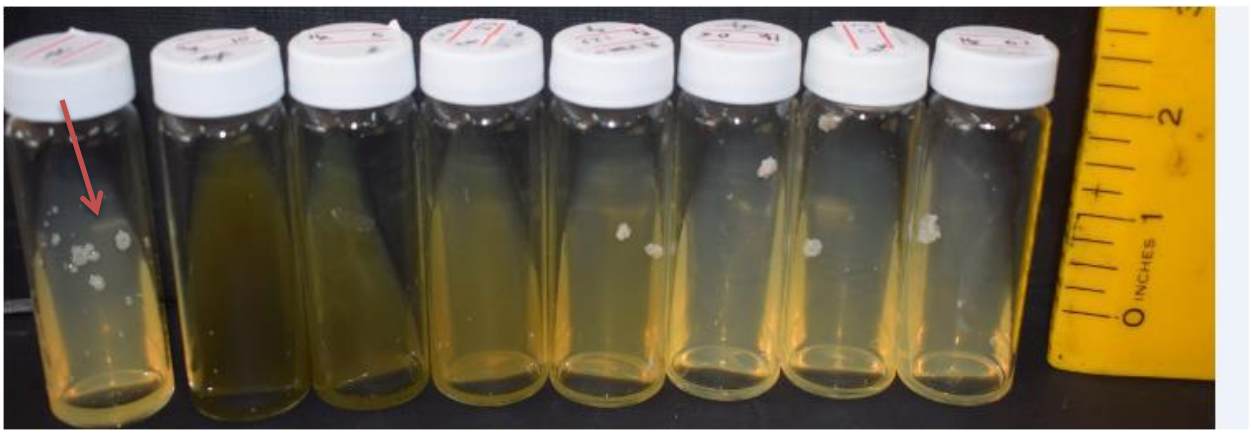
Key: x=No growth observed, += Growth observed, *= MIC

A)

10mg/mL 5mg/mL 2.5mg/mL 1.25mg/mL 0.625mg/mL 0.312mg/mL 0.156mg/mL

B)

10mg/mL 5mg/mL 2.5mg/mL 1.25mg/mL 0.625mg/ML 0.312mg/mL 0.156mg/mL



C)

0.8µg/mL 0.4µg/mL 0.2µg/mL 0.1µg/mL 0.05µg/mL 0.025µg/mL 0.0125µg/mL

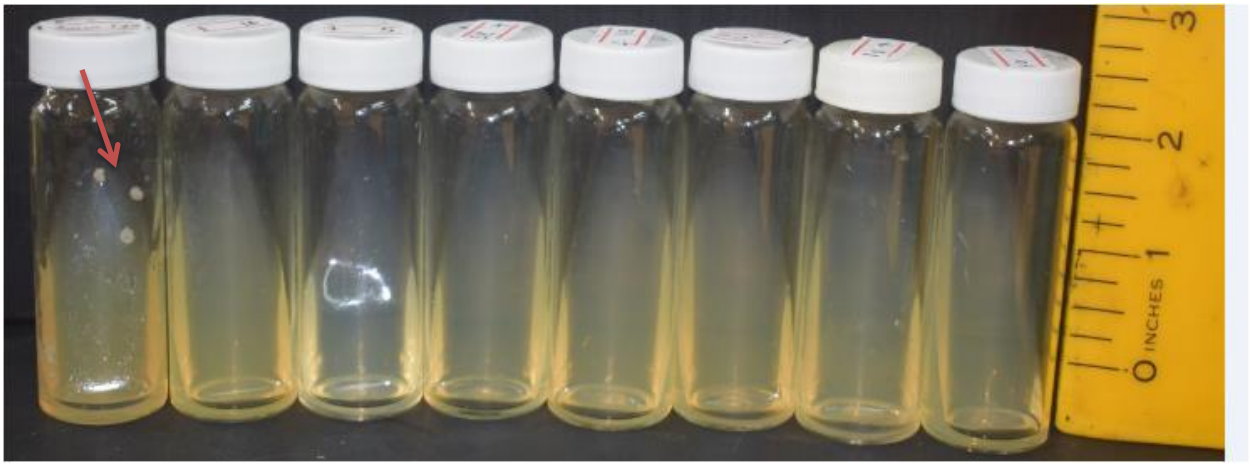


Figure 12: MIC of the plant extracts A) MIC of *A. mexicana* B) MIC of *Z. scabra* C) MIC of Ketoconazole (Positive control) and red arrows shows Negative control

4.4. Molecular Characterization of *Hcf*

Three representative mycelial isolates were selected and analyzed by semi-nested PCR. A 514-bp amplicons was observed for all of the as depicted in Figure 13 confirming the identity of the fungi to be *H. capsulatum* var. *farciminosum* since the samples were isolated from clinical cases of horses in Ethiopia.

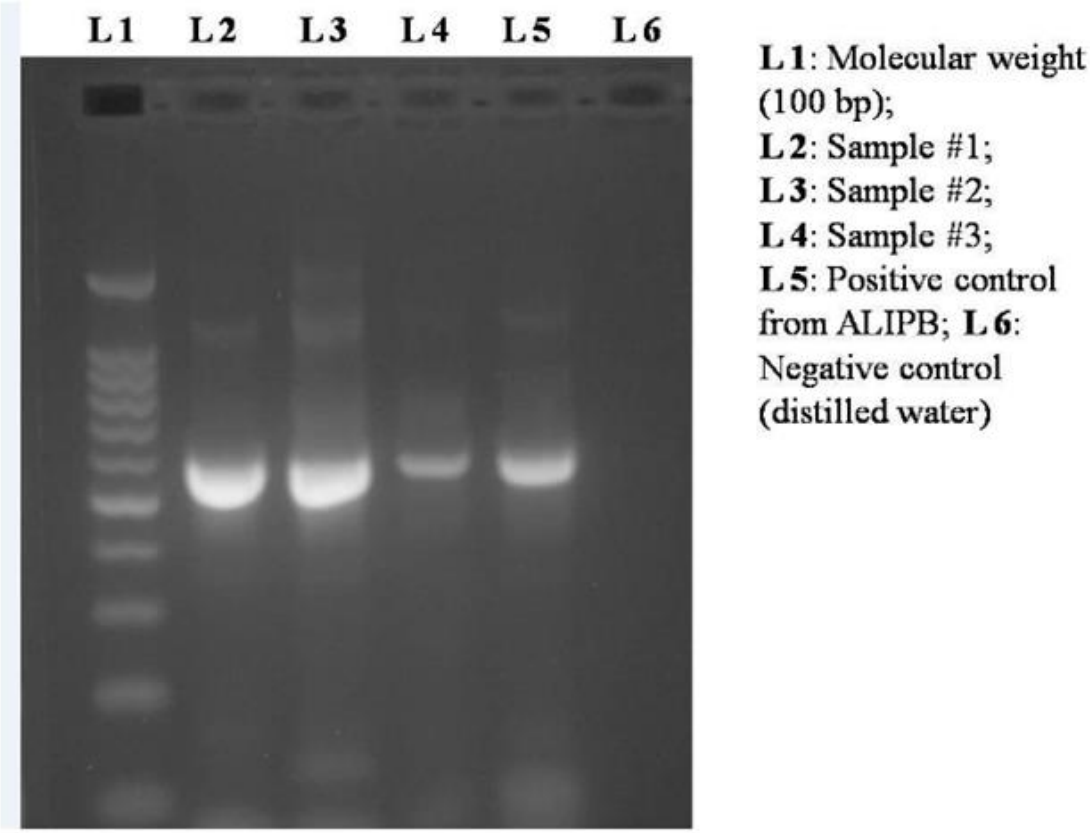


Figure 13: Gel electrophoresis of PCR amplification result gained from DNA extracted from mycelia.

DNA preparation was amplified with primers P3/2R8 (first round) and then diluted one in ten and subjected to a second round of PCR amplifications with PCR primers F5/2R5 to generate ITS gene products (51bp) indicative of the presence of Histoplasma DNA. Sample #1 represent sample taken from Shashemene, Sample #2 from Asella, Sample #3 from Gindhiir.

5. DISCUSSION

Horses provide a multitude of services to the community in Ethiopia (Hadush and Ameni, 2008) including draught power for ploughing in some parts of the country, transport and especially cart horses provides urban dwellers with the opportunity of income generation. However Epizootic lymphangitis (EL) is one of the technical factors affecting functions of horses, particularly carthorses the society gained from their cart horses is due to reduced working hours because of morbidity, reduced working life because of mortality and loss of horses due to mortality. It continued to cause economic losses due to poor response of EL to treatment, the longer duration required and treatment of EL using modern drugs is expensive for cart horse's owners. In addition to lack of effective therapeutic approach, the absence of easy, rapid and cheaper diagnostic method, which is needed for understanding of the epidemiology of the disease, has leaded to the wide spread occurrence of EL. Many efforts' are needed to tackle the economic losses caused by the disease. These include continued search for effective treatment through screening of various plants against Hcf. In addition, evaluation of histofarcin skin test provides crucial step in the understanding of the epidemiology of EL and implement control and prevention measures. So that we evaluated the diagnostic performance of histofarcin skin test antigen under field conditions for early detection of EL. Effective treatment shall be available to play a crucial role in the management of EL. There have been many studies for effective therapy for long periods of time. Hence in this study we evaluated invitro effect of A. mexicana and Z. scabra.

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 66.67% sensitivity is low. This might be due to the inaccuracy of the clinical signs in classifying of horses as infected and healthy. To this end Gram staining of pus collected from the unruptured nodules of two of the horses, which tested negative showed the involvement of gram positive bacteria that morphologically resemble *Corynebacterium pseudotuberculosis*. Those horses were clinically classified as infected. Hence, if more robust and specific tests such as molecular and serological tests are used, the sensitivity of the skin test might be improved. On the other hand the 100 % specificity of the skin observed is an indicator of its good

performance. Previous authors, however, reported a sensitivity of 90 % (Ameni *et al*., 2006). This suggests further large scale screening of animals from various geographical areas with various stages of the disease. Reactions of horses with different stages of the disease indicate the power of this test to 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).

A study done previously on evaluation of histofarcin test in Ethiopia reported 10 % false negative in endemic areas and 0% false positives results in disease free areas (Ameni *et al*., 2006). This is closer to our observation although we report 33 % false negatives and 0% false positives results in disease free areas. The false negative results could be either due to other diseases producing similar lesions as EL because the determination of the status of the diseases was based on clinical signs or due to low level antibodies to produce visible reaction. The 0% false positive results in disease free areas were due to the fact that the climate of the district is not conducive for the survival of *Hcf* as the areas are found at high altitude 2800m above sea level and its very cold area.

In vitro evaluation of the anti-*Hcf* effects of *A. mexicana* and *Z. scabra* provided promising results. We found evidence on the possible use of herbal remedies to search for optional therapy for EL. This study also demonstrated that both plants have comparable effects considering the lack of effective treatment for EL; this observation could be taken as remarkable. Even if, no study is available on the effect of *A. mexicana* on the growth of Hcf isolated from equines, methanol extracts of *A. mexicana* leaves and stems exhibit significant antifungal activity. The growth inhibition zone against fungal species tested was double the size of Amphoterecin-B (Nilesh *et al*., 2016). In parallel experiments, growth inhibition zones with stem extracts against all tested fungi were almost doubles that of Amphoterecin-B. This observation suggests that the antifungal component within the extract could successfully inhibit fungal growth. *A. mexicana* has antifungal potential, seemingly significant and comparable to that of Amphotericin B. (Nilesh *et al*., 2016). It contains several phytochemicals such as alkaloids, flavonoids, phenolics, tannins, carbohydrates, glycosides and saponins in plenty in addition to some essential amino acids and fatty acids. The alkaloids, dehydrocorydalmine and oxyberberine, isolated from A. mexicana, were found to exhibit antifungal activities against some fungal strains such as

Helminthosporium sp., *Curvularia* sp., *Alternaria cajani*, *Bipolaris* sp. and *Fusarium udum* (Singh *et al*., 2009). The ethanolic extract of leaves of *A. mexicana* is reported to have significant anti-inflammatory and analgesic activity at a dose of 200 mg/kg in mice (Sharma *et al*., 2010). It is also reported that leaf extract of *A. mexicana* is able to show significant anti-inflammatory activity in rats; the investigators (Sukumar *et al*., 1984) are in opinion that the chemical constituents of the leaf extract such as isorhamnetin-3-O-β-D-glucopyanoside, β-amyrin, cysteine and phenylalanine might be responsible for such activity. Ghosh and his group demonstrated significant wound healing activity of the test extracts that is comparable with the established drug, nitrofurazone; the tensile strength of the extract treated group was found to be higher than the latex treated group of animals on 12th post wounding day (Ghosh *et al*., 2005). *A. mexicana* is able to show significant anti-inflammatory activity in rats (Sukumar *et al*., 1984).

Although no study is available on the effect of *Z. Scabra* like that of *A. mexicana* on the growth of Hcf isolated from equines this study also demonstrated the effect of *Z. Scabra* on the growth of Hcf. The preliminary phytochemical tests of *Z. Scabra* showed the presence of alkaloid, tannin, saponins, phenol, flavonol glycosides, cardiac glycosides, phytosterol, fixed oils and fats, gums and mucilage. A dose dependent antioxidant activity was observed in case of extracts. Phenolics, tannin and flavonoid content were found to be highest. *Z. scabra* methanol extract showed maximum antioxidant activity in DPPH (Biotechnology, 2011).

The alkaloids, dehydrocorydalmine and oxyberberine, isolated from *A. mexicana*, were also found to exhibit antifungal activities against some fungal strains such as *Helminthosporium* sp., *Curvularia* sp., *Alternaria cajani*, *Bipolaris* sp. and *Fusarium udum* (Singh *et al*., 2012). The results were promising with the aqueous and methanolic extracts *Z. scabra* exhibiting interesting inhibitory activity against *C. albicans* (Desta, 1993). Flavonoids have free radical scavenging and antioxidation properties, useful for their pharmacological activities including anti-inflammatory and anticancer properties (Sharma, 2006). The results of this in vitro trial should be overlooked in attempts to search for effective treatment for EL. Both *A. mexicana* and *Z. scabra* 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 both *A. mexicana* and *Z. scabra* and might be responsible for the greater potential of this plant against Hcf. These two plant species will contribute a lot for 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.

EL studied based on clinical sign previously in Ethiopia. Number of other disease causing agent confusing clinically with EL. So should to differentiate other confusing disease showing similar clinical sign with EL by culturing on SDA and BHI agar medium and as well as demonstrating that all representative mycelial isolates were PCR positive for H. capsulatum var. farciminosum. Appropriate, reliable, and rapid diagnostic tools are mandatory both for control and prevention by veterinarians and to practice wide range of studies for more knowing of disease distribution of neglected disease.

Limitations of the Study

The invitro assessment was done on the mycelial phase of *Hcf*. But the stage that is found in the body of animal is the yeast form. It is clear that the yeast form and mycelial form did not respond to treatment alike. It is difficult to infer to treatment from mycelial growth inhibition. Therefore it needs to carry out investigation on the yeast form first.

6. CONCLUSION AND RECOMMENDATIONS

This study indicated that histofarcin skin test showed a promising result to be used as a screening test with sensitivity of 66.67 % and specificity of 100 %. Two plant species: *A. mexicana* and *Z. scabra* resulted in good inhibition effect on the growth of the mycelial phase of Hcf. That is, these plants should be considered to be important options 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 a 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 *A. mexicana* and *Z. scabra* on the yeast form of Hcf is needed

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8. ANNEXES

Annex 1: Procedure for sample collection

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

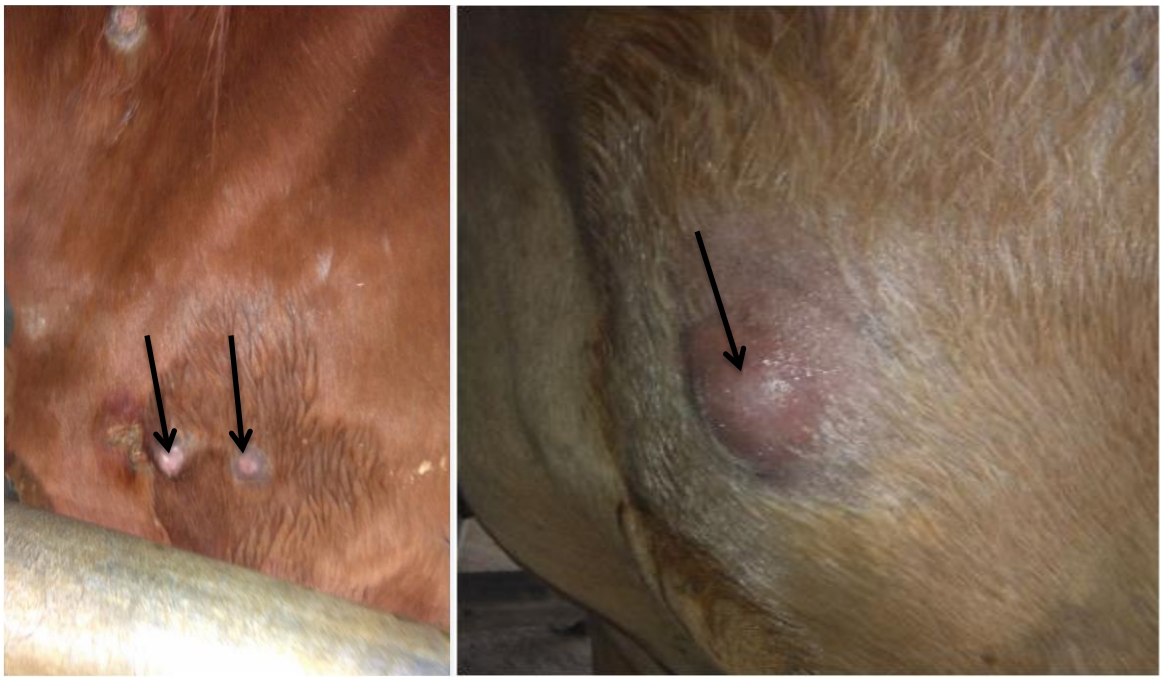

Figure 14: Washed, disinfected and shaved unruptured nodule ready for aspiration

Annex 2: procedure for preparing media and inoculation

1. A 65gm powdered 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 taken autoclaved at 121^{o} c 15 min and cooled

3. After cooling 0.5g/l chloramphenicol and 2.5% glycerol was added and gently mixed
4. The agar will be immediately poured to the bottles and kept in slant position
5. A drop of pus sample was put on the slant and striking with wire loop gently

Annex 3: Procedure for preparing Hcf antigen

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

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

Table 8: Preparation of Diluted albumin (BSA) standards

Dilution scheme for standard Test protocol and Micro plate procedure (working range = 20-2,000µg/ml)			
Vial	Volume of diluent	Volume and source of BSA	Final BSA concentration
A	0	300µl of stock	2,000µg/ml
B	125 µl	375µl of stock	1,500 µg/ml
C	325 µl	325 µl of stock	1000 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

1. Preparation of the BCA ™ working reagent (WR)

a. Use the following formula to determine the total volume of WR required: (# Standards + #unknown)X (#replicates) x (volume sample) = total volume WR required

Note: 2.0 ml of WR is required for each sample in test tube procedure while only 200 µl of WR reagent is required for each sample in micro plate procedure

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

2. Microplate procedure (Sample to WR ratio = 1:8)

a. Pipette 25 µl of each standard or unknown sample replicate in to a micropipette well (working range = 20-2,000 µl/ml).

Note: If sample size 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 was limited to 125-2,000 µl/ml).

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

c. Convert plate and incubate at 37 ^{oc} for 30 minutes.

d. Cool plate to RT

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

f. Subtract the average 562 nm absorbance measurement of the blank standard replicates from the 562 nm measurement of all other individual standard and unknown sample replicates

g. Prepare a standard curve by plotting the average blank covered 562 nm measurement for each BSA standards vs. its concentration in µl/ml. Use the standard curve to determine the protein concentration of each unknown sample.

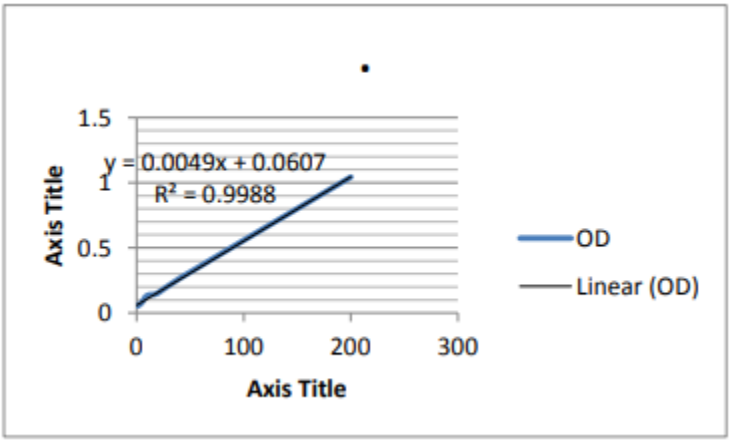

Figure 15: OD value and protein concentration graph for the histofarcin antigen

Annex 5: Procedure for plant extraction

1. Collected plants were transported to the laboratory and allowed air dry
2. Dried plant matter will be grounded by electric motor grinder and dissolved in 80% methanol at 1:6 concentration
3. The solution was allowed to stay on orbital shaker for 72hrs and it was filtered by the gauze and watman filter paper , this process was repeated three times for exhaustive extraction of particles
4. Finally methanol was evaporated by rotary vapor at 50 °c and solution was kept in an oven at 40°c for around a week to dry.

Figure 16: Grinding, mixing with 80% methanol, shaking in the orbital shaker, filtering, evaporating methanol and drying of the plant extract in the oven at 40°c