

Testing the skin with Histofarcin and examining the in-vitro antifungal properties of Croton Macrostachyus against the mycelial form of Histoplasma capsulatum Variety Farciminosum, isolated from a horse in Adama City, Ethiopia

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Abstract

Background: Epizootic Lymphangitis (EL) is a long-lasting, incapacitating condition affecting horses, which is brought about by *Histoplasma capsulatum* variety *farciminosum* (HCF). This ailment is a significant concern in the equine world, leading to both economic and welfare implications for horses.

Method: An experimental study design was conducted from Nov, 2021 to June, 2022 to examine the histofarcin skin test and assess the antifungal effectiveness of *Croton macrostachyus* against HCF mycelium. The histofarcin antigen for the skin test was derived from HCF field isolates identified in clinical cases EL in cart horses from Adama City.

Results: Histofarcin skin test was evaluated on 64 horses, 20 clinically infected cases, and 44 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 the injection and 24 hrs post injection using Bar Knight Mc Lintock(McLK)-P0005) caliper. The sensitivity and specificity of histofarcin skin test were 100% and 95% respectively. An *in vitro* growth inhibitory effect of *Croton macrostachyus* against the mycelia form of HCF was evaluated. Aculture media with no antifungal agent and media containing ketoconazole served as a negative and positive control, respectively.

Conclusions: The result showed that *Croton macrostachyus* have an inhibitory effect at concentration range from 2.5-10mg/ml. Thus, the minimum inhibitory concentration of the plant extract was found to be 2.5mg/ml. In conclusion, this study demonstrated that histofarcin skin test is a potential diagnostic tool for screening EL. Also, the methanol extracts of *Croton macrostachyus* showed good *in-vitro* growth inhibitory activity against the mycelial phase of HCF. Therefore, Validation of the performance of the histofarcin skin test assessment is necessary. Also, further investigation of the effects of *C. macrostachyus* on the yeast form of HCF is needed in addition to Phytochemical analysis (fractionation) and evaluation of the toxicity of the plant.

1. INTRODUCTION

1.1 Background and Justification

Ethiopia had the largest equine population in the world next to China [1]. It was reported that about 2.01 million horses, 8.85 million donkeys, and 0.46 million mules comprised the working equine population in Ethiopia [2].

Despite their huge numbers and significant contribution to the communities and the national economy [3], the attention given to studying the health aspects of working equids in Ethiopia is not satisfactory. Among the multiple health and welfare problems affecting working equids, infectious diseases are one of the major constraints to their productivity and work performance; this often led to high morbidity and mortality [4]. Epizootic lymphangitis (EYL) is the major infectious disease of equines, it occurs more frequently in the tropical and subtropical regions because of the suitability of the environmental

conditions for the survival of the mycelial form of *Histoplasma capsulatum variety farciminosum* (HCF), which is the causative agent of the disease [5].

The disease is endemic in north, east, and northeast Africa and in countries bordering the Mediterranean Sea, India, Pakistan, and Japan in Asia [5]. In Ethiopia, the prevalence rates varied between 0% and 39% of equids, with the rate being dependent upon the region. The highest (39%) prevalence was recorded in cart horses at Mojo, central Ethiopia while the lowest (0%) was recorded at five towns including Debre Birhan, Agaro, Dinsho, Bokoji, and Sgure from 28 towns included in the study [7]. In another study, the disease prevalence in horses was reported to be 25.1% (177/705) and 27.9% (57/204) at Woliso, 20.2% (41/203) at Sebeta and 26.5% (79/298) at Asgori, Southwestern Shoa [6]

Whereas, [7] reported, 21% of the 309 cart horses screened positive for EZL in Western Ethiopia. In the study which was assessed the disease prevalence of cart horses in Adama town, a 32.84% (132/402) prevalence rate was recorded [8]. The disease had high morbidity and low mortality (10–15%) rates [9].

The diagnosis of EZL is compromised on the clinical examination of the lesion, microscopic examination of the yeast form of HCF in pus, serological tests, and skin hypersensitivity test [10]. Cultures of HCF can also be performed from clinical lesions but is challenging and rarely attempted. Therefore, reliable and robust approaches to diagnosis are required to support clinical decision-making [11].

2. MATERIALS AND METHODS

2.1 Study Area

Clinical cases of EZL for isolation of HCF were selected in Adama City and Histofarcin skin test field trial was done in Adama City. Adama is situated 84 km east of Addis Ababa (the capital city of Ethiopia) in Oromia Regional State at a latitude of 8° 35' 00"- 8° 36' 00" north and a longitude of 39° 11'57"- 39° 21' 15" east on the escarpment of the Great Rift Valley. The town has an area of 169.9 km square and 17 kebeles[12].

2.2 Study Design

A cross-sectional experimental study design was conducted from Nov, 2021 to June, 2022 to assess the histofarcin skin test and evaluate the antifungal efficacy of *Croton macrostachyus* against the mycelium form of HCF.

2.3 Microscopic Examination of Yeast Cells and Clinical Examination of Horses

A total of 64 cart horses from Adama City displaying clinical signs of Equine Zoonotic Lymphangitis (EZL) were chosen for sampling. The selection of samples was based on the horses' susceptibility to the disease and their common parking locations. Comprehensive physical examinations were conducted to identify EZL lesions, with a particular focus on nodules on the skin. Aseptic aspiration of pus samples from unruptured nodules was performed using sterile syringes. Initially, nodules were cleaned with water

and soap, followed by shaving and disinfection of the shaved area with 70% alcohol. The prepared samples were immediately transferred into sterile brain heart infusion broth and kept cold during transportation in an icebox.

For laboratory diagnosis, smears were created from the pus before transfer into the broth at the field, using glass slides fixed with methanol and stored in a slide box. In the laboratory, the samples were stained with Wright-Giemsa stain and examined for the characteristic yeast form of *Histoplasma capsulatum* var. *farciminosum* (HCF). This form typically appears as ovoid to globose structures occurring either singly or in groups. [7].

2.4 Sample Transport and Storage

Samples collected from clinical cases were kept cool within an ice box and transported to the Veterinary Microbiology Laboratory of AHI Sebeta, refrigerated at +4 °C and processed within 24-48 hours of collection time. Positive cultures were prepared as 20% (v/v) glycerol stocks and stored at -80 °C for further analyses.

2.5 Culture Identification and Isolation of HCF

Isolation and identification of HCF were performed at Veterinary Microbiology Laboratory of AHI Sebeta. HCF was cultured according to the methods described by [13]. The aspirated pus was inoculated aseptically into slants of Sabouraud's Dextrose Agar (SDA, Oxoid) containing chloramphenicol (0.5g/l) and enriched with 2.5% glycerol. For the isolation of the mycelial form, the cultures were incubated at 26 °C with 5% CO₂ [13]. The growth media was checked periodically and Giemsa preparations were made from samples that showed growth. Brown, dry, wrinkled, and convoluted colonies were appreciated.

2.6 Confirmation of Suspected HCF Colonies by A Polymerase Chain Reaction

A sub-sample of mycelial culture isolates showing the unique morphology of HCF was confirmed by PCR amplification using ITS1 and ITS4 fungal primers.

2.7 DNA Extraction

The SDS-CTAB-chloroform-isoamyl alcohol (modified method) was employed for the extraction of DNA [21]. About 200mg of mycelia powder was taken and transferred to a 2 ml Eppendorf tube. 500 µl of SDS CTAB chloroform isoamyl alcohol (S-CCI) extraction buffer (250-mM Tris-HCl (pH 8.0), 20mM EDTA (pH8.0), 200mNaCl, 10% CTAB, 0.15% SDS) was added and vortexed. It was then boiled for 10 min at 50°C then centrifuged at 10,000 rpm for 10 min. After the centrifugation the upper liquid part was pipetted out carefully and one volume of chloroform: isoamyl alcohol (23:2) was added then mixed for 1 min and centrifuged at 10,000 rpm for 5 min. The aqueous phase was recovered and mixed with one volume of ice-cold isopropanol, and the tubes were turned upside down for 1min to precipitate the DNA. The tubes were again centrifuged at 10,000 rpm for 2 min to recover the pellet and washed with 500µl of

absolute ethanol and then recentrifuged at 10,000 rpm for 1 min. DNA was air-dried and resuspended in 200 µl deionized or TE buffer for further use.

2.8 Polymerase Chain Reaction (PCR)

A pair of primers supplied by Bioneer Corporation (8-11 Munpyeongseon-ro, Daedeok-gu Daejeon, 34302 Republic of Korea) were used for PCR reactions. The forward primer was ITS1 with sequence of 5'TCC GTA GGT GAA CCT GCG G3' while the reverse primer was ITS4 with a sequence of 5'TCC TCC GCT TAT TGA TAT GC3' [23]. The final concentration of each of the two primers was 0.1 µM/µl. PCR reaction mix (30 µl) consisting of 9 µl nuclease-free water, 3 µl of the template DNA, and 3 µl of each of primers (reverse and forward) was prepared and added into 12 µl lyophilized PCR premix (AccuPowerTaq PCR premix Bioneer, Korea) tubes which contains 1U Taq DNA polymerase, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 1x reaction buffer with 1.5 mM MgCl₂, trace amount of tracking dye, and patented stabilizer which were already aliquoted in PCR tubes and dissolved and spun down. The PCR was performed in the thermal cycler (Eppendorf Master cycler) was programmed for 1 denaturation cycle at 95 °C for 5 minutes followed by 35 cycles of the succeeding steps denaturation 95 °C for 45 seconds, annealing at 58 °C for 1 minute. And extension at 72 °C for 1 minute and a final extension period of 72 °C of 7 minutes were made [22].

2.9 Gel Electrophoresis

The PCR product was visualized using gel electrophoresis. Agarose gel was prepared using 2% agarose made in 1x TAE buffer containing red gel (2.5 µl). After loading the amplicons in each well, an electric current of 150 MA and 110 V was applied for 45 min. 1Kb DNA ladder was used as the molecular weight standard. Positive results were indicated by the presence of a 600 base pair band seen on the gel with an ultraviolet illuminator and taking the gel image using a UV trans illuminator.

2.10 Histofarcin Skin Test

2.10.1 Antigen preparation

Mycelial colonies of HCF were aseptically collected from Sabouraud dextrose agar medium, and weighed. About 2mg/mL of the fungus was thoroughly ground using a porcelain mortar and pestle in a 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 the 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 was determined by using bicinchoninic acid (BCA) protein assay as described by Pierce, BCTTM protein assay kit number 23225. A final concentration of 0.2mg/ml protein (histofarcin antigen) was used for histofarcin skin test.

2.10 Evaluation of histofarcin skin test

A total of 64 horses were used for the evaluation of the histofarcin skin test. 20 of them were clinically positive and the rest were clinically negative. The middle cervical area of the lateral side of the neck was washed with soap and water and shaved. Then after, the skin thickness was measured using a caliper. After that, 0.1 ml, which contains 0.2 mg/ml concentration of antigen was injected intradermally by using a needle having 27 gauge 1ml and 23 gauge 3ml into the middle of the neck. The thickness of the skin was measured 24 hrs post-inoculation [7]. A mule was considered to be positive when the change in the size of skin indurations at the site of administration was >4 mm.

2.11 Preparation of *Croton macrostachyus* Extract

Croton macrostachyus, leaves were collected from in and around Adama City for screening its effect against HCF (figure 1). After collection, the plant was taxonomically identified and authenticated by experts. Then, the plant was air dried and ground to make it powder form. Methanol extracts were obtained by direct dipping of dry and powdered plant material at a 1:10 ratio in 80% methanol for 48 hrs in 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 a vacuum rotary-evaporator at 50°C and to dry it completely it was kept at 40°C in a micro oven for one week for later use the stock powder was stored in deep freeze.

Serial dilutions of the stock solution were made by using 5% Dimethylsulphoxide (DMSO) as a diluent to obtain solutions with the following concentrations: 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml and 1.5625 mg/ml. Five milliliters from each of the seven dilutions were mixed with 45 ml of SDA (with 2.5% glycerol) to prepare media containing the following concentrations of the extract: 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25mg/ml, 0.625 mg/ml, 0.3125 mg/ml and 0.15625 mg/ml. After mixing well using a Vortex, each media containing the serial dilutions of the extract was dispensed into three universal bottles (30 ml capacity), allowed to solidify in an inclined position, and kept at 4 °C for 5 hours DMSO until inoculation with the test fungus. As a positive control ketoconazole (standard) was used and diluted 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. The dilutions corresponded within the range of MICs of Ketoconazole against isolates of HCF as reported by [14]. Cultures of HCF without any treatment served as negative controls.

2.11.1 Inoculum preparation and inoculation of culture media

A pure sub-culture of the mycelial colony of HCF on SDA (with 2.5% glycerol and 0.005% chloramphenicol) was used for the tests. The inoculum suspension was prepared by transferring a portion of the fungal colony to a sterile saline solution in a sterile test tube using a sterile wire loop. The turbidity of the suspension was adjusted by adding a fungal colony or saline and vigorously mixing using a vortex until turbidity approximates that of a 0.5 McFarland standard. One milliliter of 2% polysorbate 80 was added to the suspension to mix the fungal colony. The inoculum suspension was inoculated into

triplicates of the test media and the controls using sterile swabs. After dipping into the suspension, the swab was uniformly streaked all over the agar surface.

2.11.2 Determination of MIC

Minimum inhibitory concentration was defined as the lowest concentration of a drug that completely inhibited visible growth [15]. The MIC of the extract was determined after incubating the inoculated media at 27 °C for 60 days.

2.12 Data Analysis

The histofarcin skin test was evaluated by considering clinical signs and microscopy as a reference. Sensitivity was defined as the proportion of reactor (positive by the histofarcin test) horses that were positive by clinical signs and microscopic examination. Specificity was defined, according to this study, as the proportion of non-reactors that were free from the disease based on clinical signs [16]. Skin test scores were recorded and based on the value, the sensitivity and specificity together with the positive and negative predictive values were determined. The *in vitro* antifungal activity of the test media was checked for the growth inhibition of HCF in each concentration. A minimum inhibitory concentration was given for a concentration that inhibits the HCF at the end.

3. RESULTS

3.1 Isolation and Identification of HCF

A total of 64 samples were collected from cart horses of Adama City, and 31 isolates were cultured successfully. Which made the culture positivity rate 48.4. Clinical examination of cart horses from which the sample was taken revealed ulcerating skin lesions on parts of the front limbs, neck, and other parts of the skin along their lymph vessels with ruptured as well as unruptured nodules (Fig. 2).

Wright-Giemsa stained smears made from pus samples aspirated from unruptured nodules revealed the presence of double-contorted yeast cells (Fig. 3). Inoculation of the pus aspirate into BHIA (with 5% horse blood and 0.005% chloramphenicol) resulted in the growth of brown, dry, wrinkled and convoluted colonies after 2 months of incubation at 26°C. The subcultures on SDA (2.5% glycerol) grew as whitish to whitish brown, dry, convoluted colonies after 50 days of incubation at 26°C (Fig. 4).

3.2 Field Evaluation of the Histofarcin Skin Test

A total of 64 (20 clinically infected and 44 healthy) horses were used to evaluate the diagnostic performance of histofarcin skin test. Out of 20 infected horses all of them tested positive using histofarcin skin test antigen as shown in Table 1. That is, the sensitivity of the histofarcin skin test was

100% (95% CI: 81.89, 100.00). Whereas out of the 45 clinically healthy horses, only 2 of them tested positive and the rest tested negative yielding specificity of 95% (95% CI: 81.89, 100.0).

Table 1
Result of histofarcin skin test,

		Infection Status		
		Positive	Negative	Total
Histofarcin Skin Test	Positive	20	2	22
	Negative	0	43	43
	Total	20	44	64
	Sensitivity	100%		
	Specificity	95%		

3.3 Agar Dilution Assay

Five representative isolates of HCF were selected for in-vitro antifungal evaluation of the plant *Croton macrostachyus*(Fig. 5). Fungal growth was not observed in media containing the metanolic extract of *Croton macrostachyus* at concentrations of 10mg/ml, 5mg/ml, and 2.5 mg/ml. The MIC of *C. macrostachyus* was 2.5mg/ml and that of the positive control (ketoconazole) was 0.025µg/ml. The highest concentration of the extract without growth-inhibitory effects was 1.25mg/ml. HCF was grown on the media with no antifungal agents (negative control). The results of the agar dilution assays are summarized in Table 2.

Table 2
Growth of HCF in different concentrations of *C. macrostachyus* and ketoconazole

Extract of <i>C.macrostachyus</i>		Ketoconazole	
Concentration	Growth of HCF	Concentration	Growth of HCF
10 mg/ml	-	0.8 µg/ml	-
5 mg/ml	-	0.4 µg/ml	-
2.5 mg/ml*	-	0.2 µg/ml	-
1.25 mg/ml	+	0.1 µg/ml	-
0.625 mg/ml	+	0.05 µg/ml	-
0.312 mg/ml	+	0.025 µg/ml*	-
0.156 mg/ml	+	0.0125 µg/ml	+

Keys: (*) =MIC; (-) = no visible growth; (+) = visible growth

4. DISCUSSION

The observed presentation of sores on horses affected indicated a likelihood of Equine Leukocyte Adhesion Deficiency (EL), with yeast cells evident in a sample obtained from the nodule aspirate. Furthermore, the growth traits and microscopic features of both the mycelial and yeast colonies closely resembled those of Hyperkeratotic Candidiasis of Feline origin (HCF), consistent with the provided descriptions by [13]. PCR-based detection of HCC and HCF has been reported previously by using universal fungal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT G-3'). The study showed that the detection of HCF by 600 base pair (approximately) sized amplicons by using the above universal primers was in agreement with several authors. The amplification of the rRNA gene produced a 600-bp amplicon for all fungi tested in their study [17]. So far, several studies on the potential application of histofarcin skin tests were conducted and showed reproducible results [16;18;14]. All the above studies were the clinical manifestation of the disease together with microscopy as a 'Gold Standard', and in this particular study the same diagnostic approach have been applied as a 'Gold Standard'. In this study, the analysis of histofarcin skin test confirmed that it is a promising diagnostic tool to detect epizootic lymphangitis by yielding 100% and 95% sensitivity and specificity respectively.

In the past, slightly higher (90.3%) sensitivity and lower (69%) specificity were recorded in mules of endemic parts of Western Ethiopia; Whereas, 100% specificity was reported in disease-free towns of Ethiopia [16]. In another study, sensitivity and specificity of 83.3% and 80.64% were recorded in cart horses of Central Ethiopia [18]. A recent study of Histofarcin skin test in Bishoftu cart horses also reported 80% and 70% of sensitivity and specificity respectively [14] and again a year after 66.67% and 100% Sensitivity and specificity respectively were recorded at the same place [19]. However, in the current study, the diagnostic statistics were overestimated and this may be due to the smaller sample size of the field trial. In addition, the use of clinical sign as a 'Gold standard' might also yield false negative results but in this study, 0% false negative result was recorded.

Previous studies on the evaluation of histofarcin skin tests in Ethiopia reported 10% false negative in endemic areas and 0% false positive results in disease-free areas [16]. This was closer to the current observation although the author reported 0% false negatives and 4% false positives results in this study site.

The false positive results could be because while mules were incubating the disease they were able to be sensitized sufficiently by giving skin thickness reactions although no visible lesions were produced. Another justification was that the immunity after spontaneous recovery from the disease could cause sensitization of the skin to histofarcin leading to the appearance of false positives.

The evaluation of antifungal activity of *C. macrostachyus in-vitro* against mycelial form of *Histoplasma capsulatum variety farciminosum* proved its potential antifungal effect. The methanolic extracts of *C. macrostachyus* had anti-HCF effects at various concentrations in which the minimum inhibitory concentration (MIC) was found to be 2.25mg/ml. Since there is no effective treatment for EZL, this

experiment provides an option for a treatment trial. After analysis of its constituents and evaluation of its *in vivo* effects *C. macrostachyus* could be considered as a candidate for anti-HCF drug discovery.

The inhibitory effect of *C. macrostachyus* on the growth of HCF was not reported previously. Some studies demonstrated the antimicrobial effects of leaf extract of *C. macrostachyus* on different bacteria [20;21].

Antifungal activities of aqueous and methanol leaf extracts of *C. macrostachyus* against *Trichophyton rubrum*, *Trichophyton soudanense*, and *Trichophyton violaceum* were evaluated by [22]. In their study, the MIC of aqueous leaf extract of *C. macrostachyus* against *Trichophyton rubrum*, *Trichophyton soudanense*, and *Trichophyton violaceum* was 27.5, 22.5, and 27.5mg/ml respectively while the MIC of methanol leaf extract of the plant against the same fungus was 22.5, 17.5, and 25mg/ml respectively. The MIC of both extracts was much higher than our observation. The anti-HCF effect of methanol extract of *C. macrostachyus* in the current study was highly evident. This indicated the potential of the methanolic extract against HCF.

Limitations of the Study

The *in-vitro* assessment was done on the mycelial phase of HCF. However, the stage that is found in the body of the animal is in the yeast form. The yeast form and mycelial form did not respond to treatments alike. It is difficult to infer to treatment from mycelial growth inhibition. Therefore, it needs to investigate the yeast form first.

The long incubation period needed to grow HCF resulted in overgrowth by fast-growing fungal contaminants and limited the capacity for the timely harvesting and processing of pure colonies of the fungus. In addition, the current situation in the world is due to the sudden outbreak of COVID-19, after a long cultivation and harvesting period of HCF, skin test application of histofarcin was very difficult to include a larger sample size. Despite all these limitations, the study provides a preliminary finding that would help provide a significant role in treating, preventing, and controlling EZL.

5. CONCLUSION AND RECOMMENDATIONS

This study indicated that histofarcin skin test can be used for screening of EZL infected horses with a sensitivity of 100% and specificity of 95%. This study showed that *C. macrostachyus* had a strong inhibitory effect on the growth of the mycelial form of HCF. *C. macrostachyus* inhibited the growth of HCF at concentrations ≥ 2.25 mg/ml. The result indicated that *C. macrostachyus* can potentially be used for the treatment of EL provided that convenient methods of preparation, dose, and route of administration were established through rigorous *in vitro* and *in-vivo* trials. Based on these facts the following recommendations are suggested:

- Validation of the performance of the histofarcin skin test assessment is necessary with a large sample size as it showed consistent results in preliminary tests.

- Further investigation of the effects of *C. macrostachyus* on the yeast form of HCF is needed, as the yeast form is present in the host.
- Phytochemical analysis (fractionation) and the toxicity of the plant should also be investigated before study *in-vivo* trials.

Abbreviations

AAU: Addis Ababa University; ALIPB: Aklilu Lema Institute of Pathobiology; BCA: Bicinchoninic Acid; CFSPH: Center for Food Security and Public health; CSA: Centra; Statistical Agency; DACA: Drug Administration and Control Agency; DMSO: Dimethylsulphoxide; EDTA: Ethylenediamine Tetracetic Acid; ELISA: Enzyme Linked Immunosorbent Assay; EZL: Epizootic Lymphangitis; FAT: Fluorescent Antibody Technique; FTA: Flinders Technology Associates; HCC: Histoplasma Capsulatum Variety Capsulatum; HCF: Histoplasma Capsulatum Variety Farciminosum; ITC: Internal Transcribed Region; MDSA: Modified Sabouraud Dextrose Agar; MFC: Minimum Fungicidal Concentrations; MIC: Minimum Inhibitory Concentration; OIE: World Organization for Animal Health; PCR: Polymerase Chain Reaction; PDA: Potato Dextrose Agar; PPLO: Pleuropneumonia- Like Organism; SDA: Sabouraud Dextrose Agar; SDS-CTAB: Sodium Dodecyl Sulphate-Cetyl Trimethylammonium Bromide; SPANA: Society for the Protection of Animals Abroad; WHO: World health organization; YAsA: Yeast Starch Agar

Declarations

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Ethics approval and consent to participate⁴⁰⁷

The research was done based on the ethical consideration for animal and human. All methods were carried out in accordance with relevant guidelines and regulations. Ethical approval and consent for this study was obtained from Jimma University College of agriculture and Veterinary Medicine Minutes of Animal Research Ethics and Review committee (Reference AREC002/2021). Informed consent was also obtained from the farm managers to take samples from their cattle and for further research use of the samples.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from [third party]. Restrictions apply to the availability of these data, which were used under license for this study.

Competing interests

We declare that this article is original and is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Authors' contributions

Hailehizeb Tegegne: Writing- Original draft preparation, Conceptualization, Methodology, And Software.

Dese Woldegiorgis.: Data curation, Conceptualization

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Figures

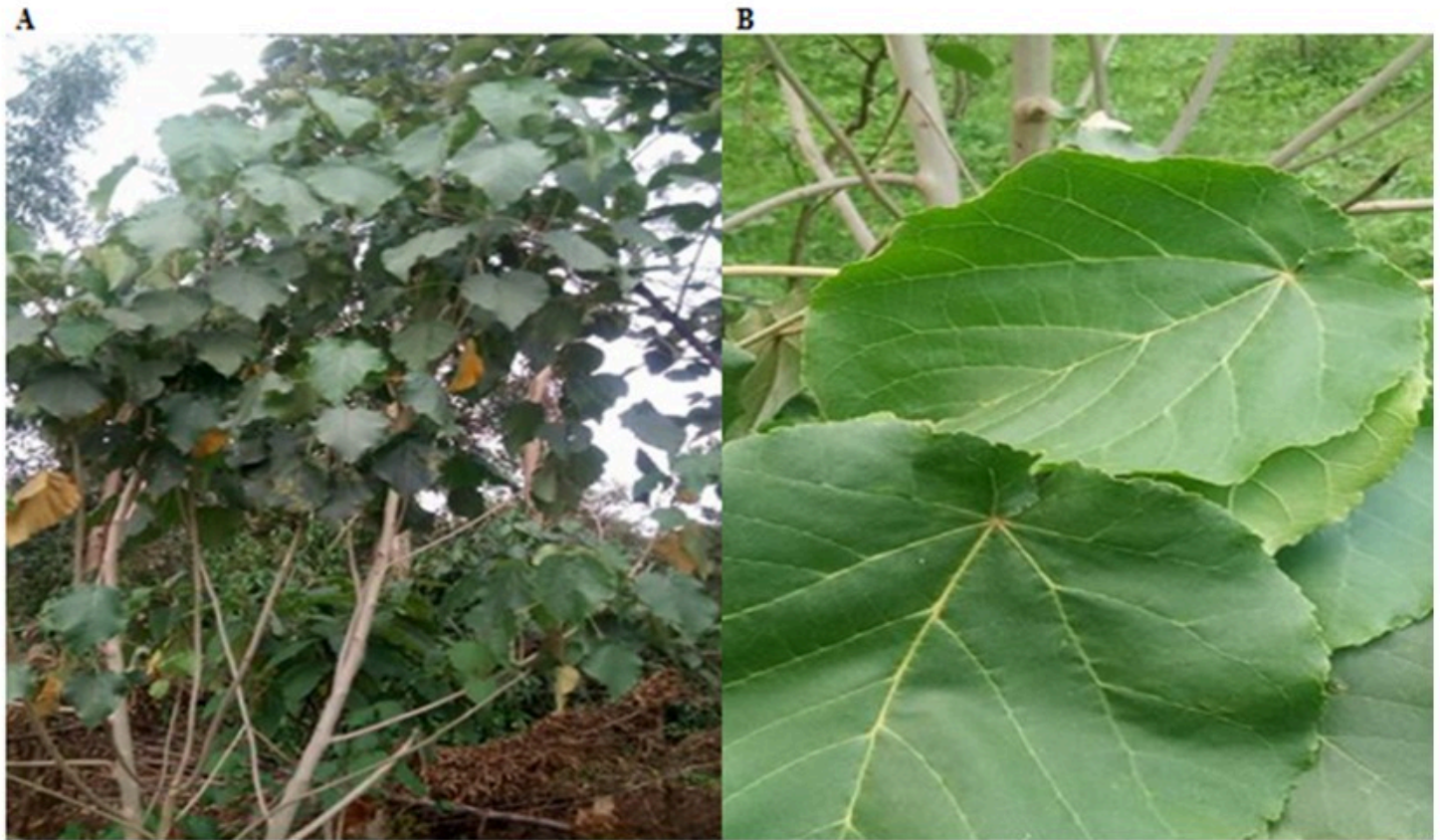


Figure 1

Tree (A) and leaf (B) of *C. macrostachyus*



Figure 2

Clinical forms of epizootic lymphangitis. A. cutaneous form in fore limb, B. pulmonary form and C. cutaneous form in hind limb [17].

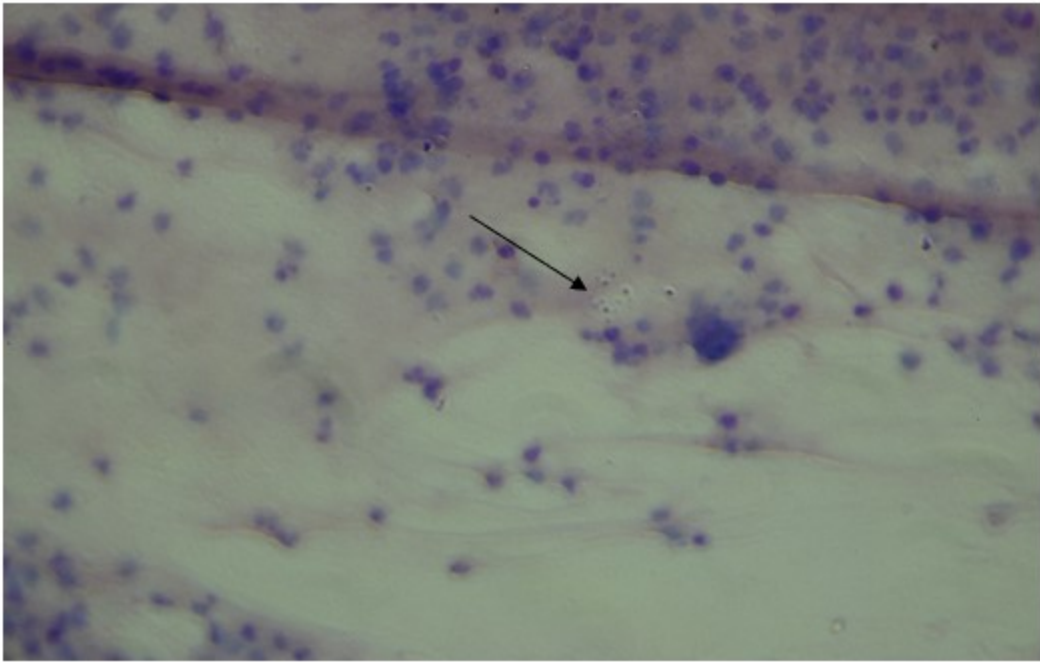


Figure 3

Microscopic appearance of HCF in Wright-Giemsa stained smear made from a pus sample demonstrating tissue form of HCF with abundant round to spherical yeast like cells (100X)

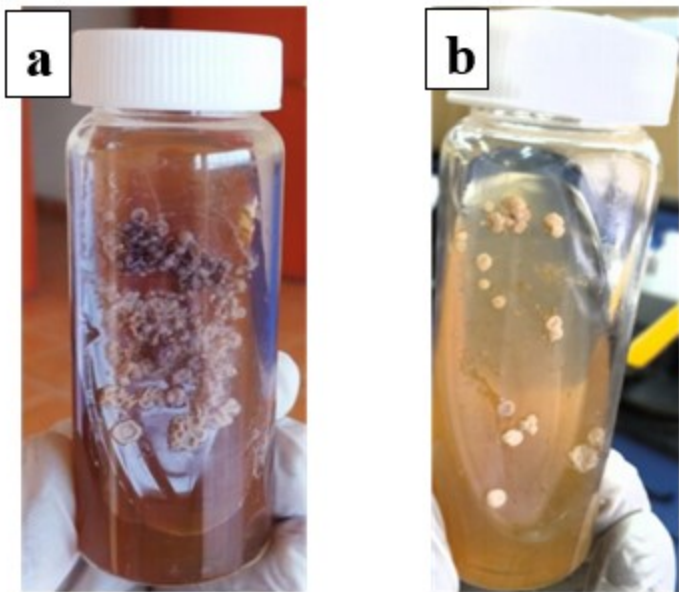


Figure 4

A primary culture of the mycelia form of HCF on BHIA with 2.5% Glycerol and 0.005% Chloramphenicol showing the brown discoloration of the colonies upon aging at 58 days of incubation (a); A pure

subculture of the mycelia form of HCF on SDA with 2.5% Glycerol and 0.005% Chloramphenicol (b) [18]

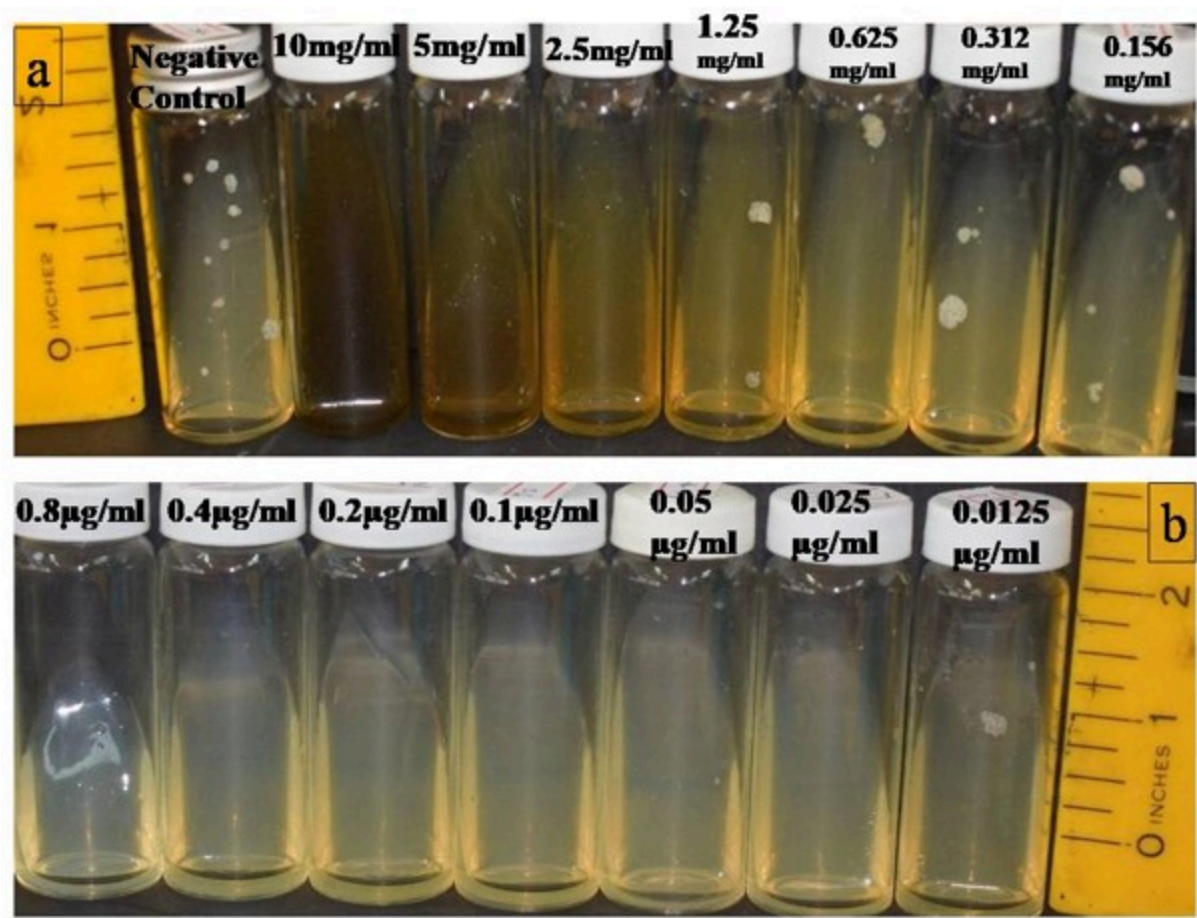


Figure 5

Agar dilution assay for *C. macrostachyus* (a) and ketoconazole (b)