

An *In-Vitro* Trial on Antifungal Effect of *Xanthium Strumarium* Leaf Extract on the Growth of the Mycelial form of *Histoplasma Capsulatum Var Farcimosum* Isolated from Horse

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Abstract

Epizootic lymphangitis (EL) has deleterious effect on both welfare and health of the horses and mules so that it has a serious negative impact on the livelihoods of cart-horse owners/drivers in the affected parts of Ethiopia. Unfortunately, antifungal drugs for the treatment of EL are costly and mostly unavailable in such areas. The aim of this study was to investigate into the effect of *Xanthium Strumarium* leaf extract (XSLE) on the Growth of the mycelial form of *Histoplasma capsulatum var Farcimosum* which has been reported to have a strong antifungal activity in previous studies, *in vitro*. *Histoplasma Capsulatum var Farcimosum* was isolated from a horse with a cutaneous form of EL. Agar dilution assay was performed to evaluate the anti HCF activity of XSLE and measure the minimum inhibitory concentration (MIC). Ketoconazole was used as a positive control. *Xanthium Strumarium* extract inhibited the growth of HCF at concentrations of 0.068 mg/ml. The result indicates that XSLE can potentially be used for the treatment of EL provided that convenient methods of preparation, dose and route of administration are established through rigorous *in vitro* and *in vivo* trials.

Keywords: Epizootic lymphangitis; *Xanthium Strumarium* leaf extract; Horses; Mule; Ethiopia

Abbreviations: MIC: Minimum Inhibitory Concentration; SPANA: Society for the Protection of Animals Abroad; HCF: *Histoplasma capsulatum var Farcimosum*; SDA: Sabouraud's Dextrose Agar; XSLE: *Xanthium Strumarium* Leaf Extract

Introduction

Histoplasma capsulatum var Farcimosum is the causal agent of epizootic lymphangitis, or preferably histoplasmosis, a chronic disease of horses and other Equidae [1] generally affecting the subcutaneous lymph nodes and the lymphatics of the neck, chest and legs but it can also be present as an ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia [2]. In all cases, the lesions are nodular and granulomatous in character, and the organism, once established, spreads locally by invasion and then via the lymphatics [3]. Epizootic lymphangitis is a contagious disease which can infect humans [4]. The yeast form of the fungus is found in the infected host and the mycelial saprophytic stage in the environment [3,5]. Epizootic lymphangitis has a serious negative impact on the livelihoods of cart horse owners/drivers in the affected areas

and also compromises the welfare of working horses [6]. The disease is endemic in countries bordering the Mediterranean, particularly in Italy and North Africa and is also found in Central and Southern Africa, and in regions of Asia and Russia [4].

The mode of transmission of the disease is not well established [7]. Direct contact with infective materials through injured skin or through cutaneous abrasions is the most common mode of infection [8]. In Ethiopia the disease was reported to have an overall prevalence of 18.8% in 28 towns being more common in mid altitude areas (1500 to 2300 meter above sea level) [9].

Recommended treatments for EL include intravenous sodium iodide or intravenous amphotericin B [4]. Another treatment regimen involves local treatment of skin lesions by incising

nodules, draining the pus and infusing with 4% iodine tincture followed by oral potassium iodide (30 g for a horse of 200–250 kg) daily for 5 days and then every other day for a further 3–4 weeks or longer if owners comply [10]. This regimen, devised by the Society for the Protection of Animals Abroad (SPANNA) clinic at the College of Veterinary Medicine and Agriculture of Addis Ababa University, was reported to result in a recovery rate of 85% [11].

Ethno pharmacologists, botanists, microbiologists, and natural products chemists are combing the Earth for phytochemicals and “leads” which could be developed for treatment of infectious diseases [12]. Medicinal plants would be the best source to obtain a variety of drugs in developed countries about 80 of plants are used in traditional medicine [13]. Over 50% of modern clinical drugs are natural product of medicinal plants and these natural products play an important role in drug development programs in the pharmaceutical industry [14] humans are using natural product of animals, plants and microbial sources for thousands of years either in the pure form or crude extracts [15].

In Ethiopia the medicinal plants are widely used in the society. This has been examined to assure the plants efficacy in treating disease by some researchers. For instance an *in-vitro* study conducted by [6] indicated that fresh garlic extract has some degree of growth inhibitory effect on the mycelial form of *Histoplasma capsulatum* var *Farcimosum* [16] has conducted evaluation of berries of *Phytolaccadecandra* for growth inhibition of *Histoplasma capsulatum* var *Farcimosum* and treatment of cases of epizootic lymphangitis in Ethiopia and that was effective.

The works mentioned above are good indicators that *Xanthium Strumarium* leaf extract may have a potent inhibitory effect on *Histoplasma capsulatum* var *Farcimosum* (HCF). There was no research that has been done so far in this plant extract to assure whether it has antifungal effect for the treatment of EL. Therefore, the objectives of this study were to investigate into the effect of *xanthium strumarium* leaf extract on the growth of HCF and to determine the minimum inhibitory concentration (MIC) *in vitro*.

Materials and Methods

Study area

The study was conducted at the College of Veterinary Medicine and Agriculture of Addis Ababa University between November 2013 and May 2014.

HCF Isolate

The *Histoplasma capsulatum* var *Farcimosum* isolate was found from Aklilu Lemma Institute of Pathobiology, Addis Ababa University in which it was isolated according to the methods described by [3]. The isolate was then sub cultured

into Sabouraud’s dextrose agar (SDA) slants (2.5% glycerol and 0.005% chloramphenicol) and incubated for 21 days at 27 °C.

Agar dilution assay

Medicinal plant used for study

***Xanthium strumarium* / rough cocklebur:** is broad leaved, tap rooted herbaceous annual plant which is in family Asteraceae, sub family asteroideae tri beheliantheae, genus xanthium and species *xanthium strumarium*. It grows as weed throughout on waste lands. Cockleburs are short day plants and they can also flower in the tropics where the day length is constant. The herb is reputed as medicine in Europe, China, Indo-china, Malaysia and America also [17]. Stem is erect, ridged, rough and hairy and frequently branched resulting somewhat bushy plants from 30-120cm tall. It has small green unisexual flower occurring in separate cluster at the end of the branches and main stems. The fruit is brown, hard, woody, bur from 0.4-0.8 inch long and covered with stout, hooked bristle. Its seed are produced in hard, spiny, globes or oval double chambered single seeded bur [18]. Beside its medicinal value, if small quantity of parts of the mature plant is consumed, the seeds and seedlings will cause intoxication because extremely toxic chemical carboxyatratyloside is contained in them [19].

Collection of medicinal plant

The Collection of medicinal plant was done in Kemissie Amhararigion of Oromia zone. Fresh plants were collected and shade then powdered. Powder was preserved in zip lock bags at room temperature.

Preparation of *Xanthium strumarium* leaf extract (XSLE)

25 grams of *xanthium strumarium* were mixed with 250 ml of 95% ethanol in separate bottle and shaken gently. After this was done the mixtures were left in the laboratory class at room temperature for 7 days. After that the solutions were filtered by filter paper into another pure jar. Then it was put in vacuum drying machine and ethanol and plant extract were separated in different container. The plant extracts were put on Petri dish and set in the incubator for 24 hours. The extract can be changed to the stock solution any time after 24 hours incubation. Therefore the stock solution was prepared after two weeks by diluting with distilled water.

Preparation of the test media

Serial dilutions of the XSLE stock solution were made by using sterile distilled water as a diluents to obtain solutions with the following concentrations: 4.91 mg/ml, 2.18 mg, 1.11 mg/ml, 0.55 mg/ml, 0.27 mg/ml, 0.14 mg/ml, 0.068 mg/ml and 0.034 mg/ml. 2 ml of distilled water was added into each 7 universal bottles and into the first universal bottle 2ml of stock solution was added. From 8% XS 2ml of the extract was taken and

dropped into the first three universal bottles. Then three serial dilutions were made for the remaining 7 universal bottles. Then into each 8 universal bottles 14ml of SDA was added and mixed well and slant was made by allowing solidifying in an inclined position and kept at 4 °C for 5 hours until inoculation with the test fungus. Then the agar dilution assay and inoculation of the test fungus were repeated three times to see the reputability of the experiment.

Preparation of the control media

Ketoconazole in the range of 0.03125 µg/ ml to 16 µg/ ml (standard) and saline water were inoculated with the test fungus. Antifungal susceptibility testing broth macro dilution testing was performed in accordance with the guidelines in NCCLS document [20].

Inoculums preparation

A pure sub culture of the mycelial colony of HCF on SDA (with 2.5% glycerol and 0.005% chloramphenicol) (Figure 1) was used for the tests. The inoculums 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 fungal colony or saline and vigorously mixing using a Vortex until turbidity approximates that of a 0.5 McFarland standard [21].

Inoculation of the media

The inoculums 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.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was defined as the lowest concentration of drug that completely inhibited visible growth [22]. The MIC was determined after incubating the inoculated media at 27 °C for 21 days for sub cultured HCF colony.

Results

HCF isolate

The *Histoplasma capsulatum* var *Farcimosum* isolate which was found from Aklilu Lemma Institute of Pathobiology, Addis Ababa University was reexamined with gram staining (Figure 2) and sub cultured in which the mycelial form of the organism grows slowly under aerobic conditions at 27°C on a media, enriched Sabouraud's dextrose agar, 2.5% glycerol and 0.005% chloramephenicol (SDA) (Figure 1) for its activeness and showing characteristic morphology of the target fungal colony. Organisms are usually surrounded by a 'halo' when stained with Gram stain [3].



Figure 1: A pure subculture of the mycelial form of HCF on SDA (with 2.5% glycerol and 0.005% chloramphenicol).



Figure 2: A gram-stained smear made from HCF isolate.



0.034 mg/ml 0.068 mg/ml!! 0.14 mg/ml 0.272 mg/ml 0.55 mg/ml 1.109 mg/ml 2.18 mg/ml 4.9125 mg/ml

Figure 3: Result of the agar dilution assay for XSLE.

Agar dilution assay

Fungal growths were not observed in media containing the XSLE at concentrations of 4.91 mg/ml, 2.18 mg, 1.11 mg/ml, 0.55 mg/ml, 0.27 mg/ml, 0.14 mg/ml, and 0.068 mg/ml (Figure 3). The MIC of the XSLE against HCF was 0.068 mg/ml (Figure 3) and that of the positive control was 0.5 µg/ ml (Figure 4). The highest concentration of XSLE without growth inhibitory effects was 0.034 mg/ml (Figure 5). The results of the agar dilution assays are summarized in (Table 1).



16 µg/ ml 4 µg/ ml 2µg/ ml 0.5 µg/ ml!! 0.125 µg/ ml 0.125 µg/ ml 0.03125 µg/ ml

Figure 4: Result of the agar dilution assay for Ketoconazole.



Figure 5: *Xanthium strumarium* plant.
Source: http://en.wikipedia.org/wiki/Xanthium_strumarium

Table 1: Growth of HCF in different concentrations of XSLE and Ketoconazole.

XSLE		Ketoconazole	
Concentration	Growth of HCF	Concentration	Growth of HCF
4.9125 mg/ml	-	16 µg/ ml	-
2.18 mg/ml	-	4 µg/ ml	-
1.109 mg/ml	-	2 µg/ ml	-
0.55 mg/ml	-	0.5 µg/ ml!!	-
0.272 mg/ml	-	0.125 µg/ ml	+
0.14 mg/ml	-	0.03125 µg/ ml	+
0.068 mg/ml!!	-		
0.034 mg/ml	+		

Note: !! : MIC; -: Novisible growth; +: Visible growth

Discussion

The isolate was checked by gram staining and sub culturing so that it was characteristic HCF both in gram staining and colony morphology as indicated in [5,8,3]. The interesting foundation that was encountered while sub culturing the HCF colony was that well visible growth has been seen in 21 days even if growth is relatively slow and most isolates require from four to eight weeks for development of characteristic colonies [4]. *Histoplasma capsulatum* var *Farcimosum* produces moderately growing, white to buff brown, velvety colonies on most mycological media at 25 to 30 °C so that it was grown with this characteristics on SDA (with 2.5% glycerol and 0.005% chloramphenicol) [21].

Whole plant of *Xanthium strumarium* as well as all parts separately is used in medicine [17]. The genus *xanthium* also possess antibacterial, antiviral, antimalarial, fungicidal, insecticidal, and cytotoxic activities against cancer cell lines [23]. The inhibitory effect of Ketoconazole is 136 times potent than XSLE on the growth of the mycelial form of HCF in which it

is more potent medicinal plant.

Antifungal activity was determined by the agar diffusion method. Test samples were diluted in Sabouraud dextrose agar followed by solidification in slanting positions. Test fungal cultures were inoculated on the slant and were incubated at 29°C for 3-7 days [24,25]. Among dermatophytes, the most susceptible strain was *microsporumcanis* which showed 50% and 60% inhibition against crude extract and ethylacetate fraction, respectively. *Aspergillus flavus* was the second most susceptible fungi, exhibited 50% inhibition against n-hexane fraction [26].

The MIC of XSLE obtained in this study was 0.068 mg/ml. However, the difference between the MIC and the maximum non inhibitory concentration (0.034 mg/ml) reported in this study suggest that the MIC is higher. The principal compounds isolated from *Xanthium strumarium* leaves were found to contain, isoxanthanol, hydroquinone, caffeyolquinic acids, xanthanol, anthraquinone, cardenolide, leucoanthocyanin, simple phenolics, triterpenoids and thiazinedione [17]. *Xanthium strumarium* produces secondary metabolites such as alkaloids, tannins, terpenoids, flavonoids, chloroform and n-hexane fractions whose activity has been demonstrated to be antifungal [27]. Antifungal activity of these molecules from *xanthium strumarium* exhibited 60% and 50% inhibition activity against the major dermatophyte fungi, *microsporumcanis* [14]. Even if the effect of XS has not been studied on HCF secondary metabolites especially Chloroform (CHCl₃) and n-hexane (C₆H₁₄) group from *Xanthium strumarium* are cytotoxic with an average 9-16 mm zone of inhibition. Further investigations are, however, necessary to explore mechanism(s) of action involved in these pharmacological activities [28].

Conclusion

My study revealed that XSLE strong inhibitory effect on the growth of the mycelial form of HCF. XSLE inhibited the growth of HCF at concentrations ≥0.068 mg/ml. The result indicates that XSLE can be included in the treatment of EL provided that convenient methods of preparation, dose and route of administration should be established through rigorous *in vitro* and *in vivo* trials

Recommendation

Based on the above conclusions the following recommendations have made

- Further studies on phyto-chemical analyses should be conducted on this plant and the active chemical should be identified.
- *In-vivo* studies must be conducted so that the safety margin, toxicity and cure rates will be known in order to use them commercially.

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