

An *In-vitro* Trial on Antifungal Effect of *Xanthium strumarium* Leaf Extract on the Growth of the Mycelial Form of *Histoplasma capsulatum Var farciminosum* Isolated from Horse

Fentahun Wondmnew, Fekadu Regassa, Yitayew Demessie, Dagmawi Yitbarek and Kalkidan Getnet

Faculty of Veterinary Medicine, University of Gondar, Gondar, Ethiopia, P.O. Box: 196

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 epizootic lymphangitis are costly and mostly unavailable in such areas. The aim of this study was to investigate the effect of *xanthium strumarium* leaf extract (XSLE) on the growth of the mycelial form of *histoplasma capsulatum var farciminosum* which has been reported to have a strong antifungal activity in previous studies, *in vitro*. *Histoplasma capsulatum var farciminosum* 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). Ketoconazol 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* trials.

Key words: Epizootic lymphangitis • *Xanthium strumarium* leaf extract • Horses • Mule • Ethiopia

INTRODUCTION

Histoplasma capsulatum var farciminosum 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 is found in the environment [5, 3].

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 (SPAN) clinic at the College of Veterinary Medicine and Agriculture of Addis Ababa University, was reported to result in a recovery rate of 85% [11].

Ethnopharmacologists, 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, 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 *farciminosum* [16] and has conducted evaluation of berries of *Phytolacca dodecandra* for growth inhibition of *histoplasma capsulatum* var. *farciminosum* and treatment of cases of epizootic lymphangitis in Ethiopia and that was effective.

The works mentioned above are good indicators that *xanthium stramorium* leaf extract may have a potent inhibitory effect on *histoplasma capsulatum* var *farciminosum* (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 the effect of *xanthium stramorium* leaf extract on the growth of HCF and to determine the minimum inhibitory concentration *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 2014 and May 2015.

Study Design: Experimental study design was conducted to assess the antifungal effect of *Xanthium strumarium* leaf extract on the growth of the mycelial form of *Histoplasma capsulatum* var *Farciminosum* isolated from horse.

HCF Isolate: The *Histoplasma capsulatum* var *farciminosum* isolate was found from Aklilu Lemma Institute of Pathobiology, Addis Ababa University, in which it was isolated according to the methods described

by OIE (2008). 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 (Fig. 5) which is in family asteraceae, sub family asteroideae, tribe heliantheae, 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 which results somewhat bushy plants from 30-120cm tall. It has small green unisexual flower occurring in separate cluster at the end of the brunches and main stems. The fruit is brown, hard, woody, bur from 0.4-0.8 inch long and coved 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 a small quantity of parts of the mature plants 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 Amhara region 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): 25grams of *xanthium stramorium* were mixed with 250ml of 95% ethanol in separate bottle and shaken gently. Then the mixtures were left in the laboratory class at room temperature for 7 days. After that the solutions were filtered by filter pepper 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 petridish and set in the incubator for 24 hours. The extract can be changed to the stock solution after 24 hours incubation. Therefore, 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 and 0.068 mg/ml and 0.034 mg/ml. 2ml 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 in 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, agar dilution assay and inoculation of the test fungus were repeated three times to see the repeatability of the experiment.

Preparation of 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 change name to clinical and laboratory standards institute document [20].

Inoculum Preparation: A pure sub culture of the mycelial colony of HCF on SDA (with 2.5% glycerol and 0.005% chloramphenicol) (Fig. 1) 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 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 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.

Determination of Minimum Inhibitory Concentration: 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 farciminosum* isolate which was found from Aklilu Lemma Institute of Pathobiology, Addis Ababa University was re



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



Fig. 2: A gram-stained smear made from HCF isolate revealed typical yeast form of the organism, which appeared as Gram-positive, pleomorphic and ovoid to globose structures

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 ¹	-
0.272 mg/ml	-	0.125 µg/ ml	+
0.14 mg/ml	-	0.03125 µg/ ml	+
0.068 mg/ml ¹	-		
0.034 mg/ml	+		

Note: = MIC, - = No visible growth + = Visible growth

examined with gram staining (Fig. 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% chloramphenicol (SDA) 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].



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

Fig. 3: Results of the agar dilution assay for XSLE



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

Fig. 4: Results of the agar dilution assay for Ketoconazole



Fig. 5: *Xanthium strumarium* plant

Source: [23]

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. The MIC of the XSLE against HCF was 0.068 mg/ml and that of the positive control was 0.5 µg/ml (Fig. 4). The highest concentration of XSLE without growth inhibitory effects was 0.034 mg/ml. The results of the agar dilution assays are summarized in Table 1.

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 Weeks *et al.*, (1985), Selim *et al.*, (1985) and OIE (2008). 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. *farcinosum* produces moderately growing, white to buff brown, velvety colonies on most mycologic 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 [24]. 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 [25, 26]. Among dermatophytes, the most susceptible strain was *microsporum canis* 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 [27].

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 [28]. Antifungal activity of these molecules from xanthium strumarium exhibited 60% and 50% inhibition activity against the major dermatophyte fungi, *microsporum canis* [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 [29].

CONCLUSIONS AND RECOMMENDATION

The study revealed that XSLE strong inhibitory effect on the growth of the mycelial form of HCF. XSLE inhibited the growth of HCF at concentrations \geq 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. 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.

ACKNOWLEDGEMENTS

First of all I praise nobody but *God* and his *Mother Virgin Merry* for helping me being in front of me to cover me from evil actions and impediments in each fraction of seconds.

I would like to thank Dr. Fekadu Regassa, my advisor, for allowing me to do the thesis in my area of interest and for providing me with intellectual and material support.

I would also like to thank Dr. Mesfin Mamo for his inspiring intellectual and moral support throughout this research and Dr. Gobena Ameni too with him for giving me HCF isolate at Akililu Lemma Institute of Pathobiology, of Addis Ababa University. My glorious thanks also go to my role model Dr. Yilikal Asfaw for his intellectual and moral support throughout my study.

I would like to give my thanks for all my family and especially my brother Temesgen and sister Addis for all their electrifying moral support.

My heartfelt goes to my whole beloved friends especially my girl friend Mieraf who made my life easy with my eventful study.

REFERENCES

1. The Center for Food Security and Public Health, 2009. Epizootic Lymphangitis. Iowa State University College of Veterinary Medicine, pp: 1-4.
2. Chandler, F.W., W. Kaplan and L. Ajello, 1980. A colour atlas and textbook of histopathology cases of *histoplasmosis farciminosi* in horses and its morphological characteristics, Wolfe Medical Publications Ltd., London, 13: 507-508.
3. OIE, 2008. Terrestrial Manual for Epizootic Lymphangitis, Office International des Epizooties, pp: 852-857.
4. Al-Ani, F.K., 1999. Epizootic lymphangitis in horses: a review of the literature. Revue scientifique et technique (international Office of Epizootics), 18: 691-699.
5. Weeks, R.J., A.A. Padhye and L. Ajello, 1985: *Histoplasma capsulatum* variety *farciminosum*. a new combination for *Histoplasma farciminosum*. Mycologia, 77: 964-970.

6. Mesfin, M., 2012. The effect of fresh garlic extract on the growth of the mycelial form of *histoplasma capsulatum* var *farcimosum* - an *In Vitro* Trial(unpublished DVM thesis at College of Veterinary Medicine and Agriculture, Addis Ababa University).
7. Singh T., 1995. Studies on epizootic lymphangitis I. Modes of infection and transmission of equine histoplasmosis (epizootic lymphangitis). Indian J. Vet. Sci., 35: 102-110.
8. Selim, S.A., R. Soliman, K. Osman, A.A. Padhye and L. Ajello, 1985. Studies on histoplasmosis farciminosii (epizootic lymphangitis) in Egypt. Isolation of *Histoplasma farciminosum* from cases of histoplasmosis farciminosii in horses and its morphological characteristics, Eur. J. Epidemiol., 1: 84-89.
9. Ameni, G., 2006. Epidemiology of equine histoplasmosis (epizootic lymphangitis) in carthorses in Ethiopia, The Veterinary Journal, 172: 160-165.
10. Jones, J., 2006. Epizootic lymphangitis: the impact on subsistence economies and animal welfare, The Veterinary Journal, 172: 402-404.
11. Aklilu, N. and A. Zerfu, 2010. Socioeconomic impact of epizootic lymphangitis (EL) on horse drawn taxi business in central Ethiopia, Proceedings of the 6th International Colloquium on Working Equids, pp: 83-86.
12. Cowan, M.M., 1999. Plant products as antimicrobial agents, Clinical Microbiology Reviews, 12: 564-582.
13. World Health Organization, 1997. Quality assurance of pharmaceuticals: a compendium of guidelines and related materials. Geneva, 1: 31-37.
14. Bharathi, B., R. Padmini, D. Swamidoss and Gorge, 2010. Phytochemical analysis and antibacterial activity of *solanum trilobatum* against HIV associated bacterial pathogens. Int J.P.H. Sci., 2(2): 598-605
15. Parekh, J. and S. Chanda, 2007. *In-vitro* antimicrobial activity of *Trapa natans* Linn. Fruit rind extracted in different solvents. Afr. J. Biotechnol., 6: 766-770.
16. Negesse, M.N., M. Eyasu, A. Nigatu and A. Gobena, 2012. evaluation of berries of *phytolacca dodecandra* for growth inhibition of *histoplasma capsulatum* var. *farcimosum* and treatment of cases of epizootic lymphangitis in Ethiopia. Aiasian Pac. J. Trop Biomed, 2(7): 505-510.
17. Bhogaonkar, P.Y. and S.A. Ahmad, 2012. Pharmacognostic studies on *Xanthium strumarium*. A Folk Unani Medicinal Herb, Bioscience Discovery, 3(1): 101-106.
18. Agharkar, S.P., 1991: Medicinal plant of Bombay precidency. pbl. scientific publishers, Jodhpur (India), 1: 852-857.
19. Madalln, R.L. and J.P. Sing, 2001: Diseases, competitors moulds and physiological disorders of button mushroom and their management, Journal of Chafan Singh Haryana, 30(1): 193-194.
20. Ren-kai, L., A.C. Meral, N. Nicole, P. Lester, W.W. David and R.M. Michael, 2000. Antimicrobial agents and chemotherapy, American Society for Microbiology, 44(6): 1734-1736.
21. Deanna, A., A. Aarvind and P.G. Padhye, Standard and G.R. Michael, 1997. An aberrant variant of *histoplasma capsulatum* var. *capsulatum*. American Society for Microbiology, Journal Of Clinical Microbiology, 35(3): 734-735.
22. Li, R.K., M.A. Ciblak, N. Nordoff, L. Pasarell, D.W. Warnock and M.R. McGinnis, 2000: A clinical and laboratory update, Journal of Epidemiology, 1: 84-89.
23. http://en.wikipedia.org/wiki/Xanthium_strumarium, accessed May 28, 2014.
24. Sravani, P.S., L. Mohana and A.S. Kumar, 2010. Evaluation of Diuretic Activity of *Xanthium strumarium*, International Journal of Preclinical and Pharmaceutical Research, 1(1): 31-34.
25. Paxton, J.D., 1991. Assay for antifungal activity. Academic Press, London. Wolfe Medical Publications Ltd. London, England. new combination for *Histoplasma farciminosum*. Mycologia, 77: 964-970.
26. Nisar, M., W.A. Kaleem, M. Qayum, H. Abrar, M. Zia-ul-haq, I. Ali and Choudhary M.I, 2010: Biological screening of *zizyphus oxyphylla* Edgew leaves. Pak. J. Bot., 42: 4063-4069.
27. Fazli, K., I. Zafar, K. Ayub, F. Zakiullah and S.K. Muhammad, 2012. validation of some of the ethnopharmacological uses of *xanthium strumarium* and *duchesnea indica*, Pak. J. Bot., 44(4): 1199-1201.
28. Gujar, J. and D. Talwankar, 2012. Antifungal potential of crude plant extract on some pathogenic fungi, World Journal of Science and Technology, 2(6): 58-62.
29. Nasrin Fatema, 2014. Antimicrobial and cytotoxic activity of ethyl acetate, chloroform and n-hexane extracts of *cucumis sativus* leaves, International Journal of Science and Technology, 21(3):1480-1486.