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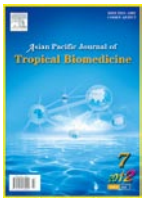

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Evaluation of berries of *Phytolacca dodecandra* for growth inhibition of *Histoplasma capsulatum* var. *farciminosum* and treatment of cases of epizootic lymphangitis in Ethiopia

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ABSTRACT

Objective: To evaluate the berries of *Phytolacca dodecandra* (*P. dodecandra*) for its effect on *Histoplasma capsulatum* var. *farciminosum* (HCF) and for the treatment of cases of epizootic lymphangitis (EL). **Methods:** Samples were collected from un-ruptured nodules of cases of EL at Debre Zeit and Akaki (central Ethiopia). Mycological culture and isolation of HCF were performed at the Aklilu Lemma Institute of Pathobiology. Phytochemical screening was done for *n*-butanol extract of *P. dodecandra* to detect alkaloids, saponins, phenolic compounds and flavonoids. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of aqueous and *n*-butanol extracts of *P. dodecandra* against HCF were determined by agar dilution assay. For the *in vivo* trial, 5% simple ointment was prepared from *n*-butanol extract and applied topically to 24 (twelve early and twelve moderate) cases of EL. **Results:** Phytochemical screening showed that *n*-butanol extract of *P. dodecandra* was positive for alkaloids, saponins and phenolic compounds but negative for flavonoids. The MICs of *n*-butanol and aqueous extracts of *P. dodecandra* were (0.039%-0.078%) and (0.625%-1.250%), respectively. The MFCs of *n*-butanol and aqueous extracts of *P. dodecandra* were (0.078%-0.156%) and (1.250%-2.500%), respectively. The MIC and MFC of ketoconazole (positive control) was (1.200×10^{-5}%-2.500×10^{-5}%) and (5.000×10^{-5}%-1.000×10^{-4}%), respectively while growth was observed on free medium (negative control). From the total of 24 treated cases of EL, 14 (58.3%) responded to treatment; however, 10 (41.7%) did not respond to treatment. There was no significant difference in the degree of response to treatment between early and moderate cases (χ^2=0.686; P=0.408). **Conclusions:** It can be concluded that *n*-butanol extract of *P. dodecandra* demonstrates antifungal effects while the aqueous extract shows no antifungal activity.

1. Introduction

Epizootic lymphangitis (EL) is a contagious, chronic disease which mainly affects horses, mules and donkeys. It is caused by *Histoplasma capsulatum* var. *farciminosum* (HCF). The disease is characterized clinically by a suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis. It is seen most commonly in the extremities, chest wall and the neck, but it can also be manifested as an ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia. The organism may also invade open lesions including ruptured strangles abscesses and castration wounds[1]. EL is more common in tropical and subtropical regions than in temperate zones. Currently, HCF is endemic in some countries in the Mediterranean region, and in parts of Africa and Asia including India, Pakistan and Japan[1]. HCF infects animals through wounds. The source of the organisms can be the skin lesions, nasal and ocular exudates of infected animals, or the soil. This organism can also be spread by fomites and biting flies mechanically.

Many treatment types have been tried, largely without success. Parenteral iodides and amphotericin B have been reported as effective. However, although the disease is highly prevalent and economically important in Ethiopia[2], the treatment options mentioned have not been employed because of the cost of the drugs and their absence in Ethiopia. This warrants for the need for other approaches

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including the use of traditional remedies. In Ethiopia, traditional medicine has long been practiced. It is common for Ethiopians to treat some common ailments using plants available around them[3]. Natural products and their derivatives have historically been sources of therapeutic agents.

As drugs such as aspirin, digitalis, morphine, and quinine were all originally isolated or synthesized from compounds derived from plants, knowledge gained from the use of medicinal plants and their active ingredients serves as the foundation for much of modern drugs[4]. Antifungal effect is one of the effects of secondary metabolites produced by plants. *Phytolacca dodecandra* (*P. dodecandra*) is one of the many plants claimed to have antifungal secondary metabolites. Many studies indicated that, saponins are responsible for its antifungal effect. The antifungal effect of the crude aqueous extract of *P. dodecandra* was demonstrated *in vitro* against different genera of dermatophytes of human pathogen and four clinical isolates of *Candida albicans*[5]. The crude aqueous extract was also found to have effect against HCF both *in vitro* and *in vivo*[6,7]. Further evaluation of different extracts of *P. dodecandra* on HCF isolates and cases of EL would of paramount importance towards the effort made to control EL in endemic countries. The objective of the present study was, therefore, to investigate the *in vitro* and *in vivo* effects of *P. dodecandra* on HCF isolates and cases of EL.

2. Materials and methods

2.1. Test fungus

Pus samples were collected directly aseptically from un-ruptured nodules of EL cases of horses visiting Society for the Protection of Animals Abroad (SPANA) Veterinary Clinic with sterile disposable syringe after washing, shaving and disinfection of the area, and placed in an icebox. Then, the samples were transported to Aklilu Lemma Institute of Pathobiology (ALIPB) Laboratory by keeping the chain cold and cultured immediately within a day[8]. The pus samples were inoculated onto Sabourauds dextrose agar supplemented with 2.5% glycerol and 0.5 g/L of chloramphenicol. Thereafter, the inoculated medium was incubated at 27 ℃ for 6-8 weeks, and the growth of the fungal colony was checked continuously once a week. Then the primary colonies were sub-cultured to get pure colony.

2.2. Collection and preparation of P. dodecandra

The berries of *P. dodecandra* (type 44) were harvested in fully developed green stage collected from ALIPB[9]. The dried berries were garbled and grinded to powder for extraction. The powder was subjected to sieve with 250 μm size mesh to get a fine material and then stored at room temperature in dry place until use.

A known weight of the powdered *P. dodecandra* was defatted with petroleum ether (RFCL Limited, New Delhi), then the extract was removed and the marc was allowed to dry. The dried marc was extracted with *n*-butanol (Blulux Laboraory Pvt. Ltd., India) using maceration method of extraction. Maceration continued for 48 hours with frequent agitation and the resulting supernatant was filtered using filter paper (Whatman No. 1). The process of extraction was repeated three times and the filtrates of all portions were collected in one vessel. The organic solvent was removed by evaporation using Rota Vapor (BÜCHI Rota-vapor R-205). The residue was then placed on a water bath at 40 ℃. In a similar way, the aqueous extract was prepared by soaking the defatted powder in the water after which, it was filtered with sterile muslin cloth and then freeze dried using lyophilizer. The resulting dried mass was weighed as percentage yield and packed into a glass vial and stored in a desiccator over silica gel until use. Fresh stock solution was prepared for the experiment whenever required[10].

2.3. Phytochemical screening

The *n*-butanol extract of *P. dodecandra* was subjected to phytochemical screening using a standard screening procedure since it showed better antifungal activity[10].

2.3.1. Test for alkaloids

Two grams of the *n*-butanol extract was stirred with 10 mL of 1% HCl and heated for 30 minutes in a steam bath. The mixture was then filtered with filter paper and 5 drops of Dragendorff's reagent were added. Turbidity with yellow-orange precipitate was concluded to indicate the presence of alkaloids.

2.3.2. Test for saponins

Two grams of *n*-butanol extract was stirred with 20 mL of distilled water. The mixture was heated in a steam bath for 5 minutes and filtered with filter paper. Ten millilitre of the filtered solutions were taken in 25 mL measuring cylinder and shaked vigorously. Formation of honey comb froth which persists upon warming was taken as a preliminary evidence for the presence of saponin.

2.3.3. Test for phenolic compounds

Two millilitre of 1% *n*-butanol extract solutions and 3 drops of a mixture of 1 mL of 1% $FeCl_3$ and 1 mL of 1% $K_3Fe(CN)_6$ were mixed. Formation of green blue colour was taken to indicate the presence of phenolic compounds.

2.3.4. Test for flavonoids

Two millilitres of 1% *n*-butanol extract solutions and 5 drops of 2% lead acetate solutions were mixed. The development of yellow orange colour was taken as an indication for the presence of flavonoids.

2.4. Evaluation of in vitro antifungal activity of P. dodecandra

2.4.1. Agar dilution assay

Agar dilution assay is one of the methods used to test the antifungal effect of natural products[11]. In this study, a stock solution of the extracts (aquoeus and *n*-butanol), was prepared in a saline as 20% concentration with 10 mL volume, by mixing 8 mL saline with 2 g of the extract. The stock solutions were then filtered with a filter paper (Whatman No. 1). For positive control, ketoconazole was used since amphotorcin B was not available in the market. Ketoconazole (with 99.1% potency) was prepared in the range