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Research Article

ANTIFUNGAL ACTIVITY OF *USNEA VARIETIES*

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ABSTRACT

Objective: Antifungal activity of total ethanolic extract and fractions of *Usnea* varieties were proved against *Aspergillus niger*, *Penicillium digitatum,* and *Rhizopus nigricans* fungi.

Methods: To perform the study of relative antifungal activity (AFR), fluconazole and ketoconazole were used as standards. The inhibition coefficient 50 (IC_{50}) for each extract and the fractions was determined.

Results: It was established that the highest activity was presented by the acetone fraction with a value of 58.53. In the study of antifungal activity, the fraction that presented the best activity against *P. digitatum* was ethanolic with a value of 44.33, while for *R. nigricans* was petrol extract, with a value of 75.35 and finally for *A. nige*r was the total extract with a value of 35.48.

Conclusions: Comparing the values obtained from the extract and the different fractions resulted in the dichloromethane fraction showing the best values.

Keywords: *Aspergillus niger, Penicillium digitatum, Rhizopus nigricans*, Relative antifungal activity AFR, *Usnea* varieties.

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INTRODUCTION

Plants produce a diverse assortment of organic compounds, the majority of which do not participate directly in growth and development of the plant. These substances traditionally referred as secondary metabolites; there functions many of which remain unknown. On the other hand, metabolites products such as phytosterols, lipids, nucleotides, amino acids, and organic acids are found in all plants and perform an essential and evident role in growth and development. The secondary metabolites are distributed among limited taxonomical groups within the plant kingdom [1]. Lichens are complex plant individuals thought to be derived from higher or lower fungi and unicellular algae. Naturally, they have the ability to establish a symbiotic relationship with an algae or cyanobacteria (phycobiont) and a fungus (mycobiont), the result of this symbiosis generates the production of secondary metabolites by the mycobiont from the carbohydrates it receives from the photobiont, through photosynthesis. These individuals store and produce phenolic compounds with high concentrations where more than 1000 active substances produced have been recognized, which are mostly produced as a chemical defense against their predators and parasites. As a worldwide spread consortium of self-supporting associations, lichens represent a symbiotic association between the photobiont (algae) and the mycobiont (fungi). Lichens grow anywhere and on anything covering approximately 8% of Earth's surface [2]. These lichens are slow-growing organisms that occupy harsh environments of life. This property of lichen marks it as pollution, indicating organisms and the best biomonitors of air quality [3]. Lichens carry thousands of secondary chemicals in them, and over the past two decades, there has been a growing interest in lichens as a source of novel and pharmacologically active biomolecules [4]. The use of lichens as folk medicine has been reported across the world for centuries, particularly in temperate and arctic regions due to its nutritive value and impressive medicinal properties [5]. In traditional medicine, lichens have multiple uses; In many countries in Europe, numerous species are used in the treatment of stomach diseases, diabetes, pulmonary tuberculosis, cancer treatment, and among others [6]. Green medicine texts include genera of lichens with medicinal properties. Such is the case of *Cladonia*, *Evernia*, *Lobaria*, *Parmelia*, *Peltigera*, *Pertusaria*, *Physia*, *Rocella*, *Usnea*, and *Xanthoria*, one evidence is that during the middle ages, lichens were prioritized in many herbaria for treatment of different diseases.

The genus *Usnea* grows throughout the temperate zones of the northern hemisphere, especially in the sub-arctic and coastal tropical forests of Europe, Asia, and North America. Species belonging to the genus have traditionally been used for pain relief and fever control, as well as being effective in tuberculosis as well as other lower respiratory tract infections [7]. Likewise, the usnic acid present in this genus was used as an ointment for wounds and burns, with greater effectiveness than penicillin. [8]. Some reports suggest that the main biochemical characteristics that may have antimicrobial activity and that are part of the secondary metabolism of the lichen species in terms of its production are mainly focused on the synthesis of products such as some acids; as well as the production of some depsidones and depsides to a lesser extent [9].

It is known that within the characteristic properties of the activity of usnic acid is its antibacterial activity against some microorganisms such as *Pneumococci*, *Streptococci*, *Staphylococci*, and some genera of mycobacteria; in addition, it is known that its concentration varies according to the type of genus that be studied since not all lichens produce the same amount of metabolite, so it can also vary according to the conditions in which the organism is usually found [10].

METHODS

Extraction

Herbs dried in the oven were pulverized into powder [11-13]. The *Usnea* varieties were pulverized in a processor to a particle size suitable for the extraction processes. It was extracted with ethanol 96%, 3 times repeatedly, using the reflux method. Subsequently, each extract was concentrated in a rotary evaporator until viscous form was obtained and they were kept at 4°C for further analysis. Six hundred grams were taken and by means of "Soxhlet" extractor, a total ethanolic extract was obtained which was fractionated by liquid/liquid with petroleum ether, dichloromethane, acetone and ethanol, and concentrated under reduced pressure in a rotary evaporator, allowing evaporate to dryness at room temperature. Total ethanolic extract was fractionated by liquid/liquid technique with solvents of increasing polarity: Hexane, chloroform, and ethyl acetate.

Antifungal activity

Test microorganisms. The microorganisms will be selected considering their development in conventional culture media, their use in antibiotic susceptibility tests, as well as their medical importance in diseases since they produce mycotoxins that are secondary metabolites present in fungi [14]. Antifungal activity of *Aspergillus niger*, *Penicillium digitatum,* and *Rhizopus nigricans* was evaluated in this study. Where the halo produced by the inhibition of the fungus growth and by the activity of total ethanolic extract or fractions of different polarity was measured, evaluation carried out in a period of 5–10 days. Gel diffusion method using plates. The antifungal evaluation was carried out with the total ethanolic extract and the fractions of petroleum ether, dichloromethane, acetone, and ethanol of the *Usnea* varieties, using the plate drilling gel diffusion method. Previously having Petri dishes with 25 ml of Sabouraud dextrose agar (ASD), 0.1 ml of the inoculum of the fungus was sown in each box on the surface of the agar, distributing it homogeneously, allowing it to penetrate for 15 min, the perforations are made in the Petri dish with a diameter of 6 mm and a depth of 7 mm in height, each perforation was sealed with 20 µl of agar, and then the sample was added for analysis. The solutions to the problem were prepared by taking 100 mg of extract or fraction in one ml of DMSO, and volumes of 30 µL, equivalent to (3 mg), 60 µL, equivalent to (6 mg), and 90 µL, equivalent to (9 mg). As a positive control of the test carried out with *A. niger*, *P. digitatum,* and *R. nigricans*, the antifungal fluconazole and ketoconazole were used, which were prepared with 2 mg dissolved in 5 mL of DMSO, and volumes of 10 µl (equivalent to 0.004 mg), 20 µl (equivalent to 0.008 mg), 30 µl (equivalent to 0.012 mg), and 40 µl (equivalent to 0.016 mg). Once the samples were applied, they were left in prediffusion for 30 min at room temperature, then they were incubated at 22°C and the inhibition diameters were measured at 5 days for *P. digitatum* and at 10 days for *A. niger* and *R. nigricans*. The growth of the halo (mm) was plotted against the mass used of the total extract and fractions (mg) of the *Usnea* varieties, obtaining by linearity the critical mass with the equation:

$$y = am + b.$$

IC_{50} values were obtained by replacing (y) with 50 and thus calculating the critical mass by means of a percentage inhibition analysis, versus the necessary concentration of the extracts of each of the fractions and total extract.

RESULTS

The growth inhibition potential of *A. niger*, *P. digitatum,* and *R. nigricans* was determined against the extracts of petroleum ether, dichloromethane, acetone, ethanol, and total extract (Figs. 1-3).

Antifungal activity of *Usnea* varieties against *P. digitatum.*

Antifungal activity of *Usnea* varieties against *R. nigricans*.

Antifungal activity of *Usnea* varieties against *A. niger*.

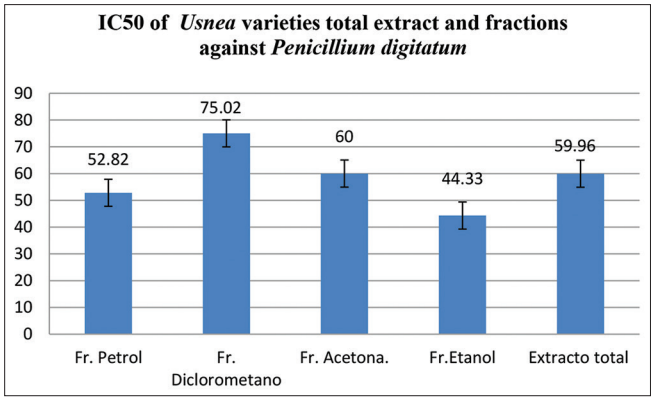

Fig. 1: Results of IC_{50} of *Penicillium digitatum* where the most favorable result with ethanol fraction (44.33) since it is the lowest and presents better activity

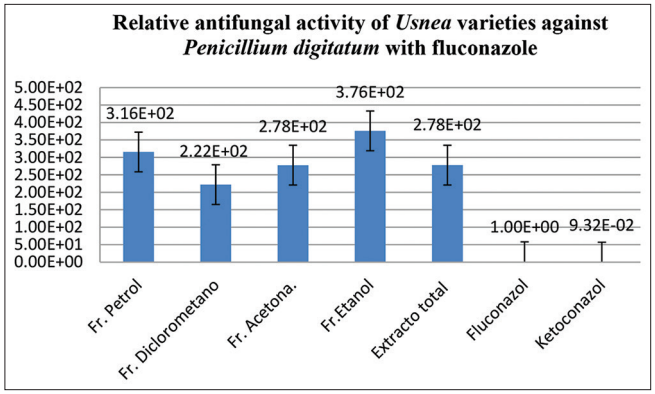

Fig. 2: *Penicillium digitatum* relative antifungal activity (AFR) with fluconazole, where when fluconazole activity was 1 mg DMSO the activity of each fraction and total extract of *Usnea* varieties, was bigger

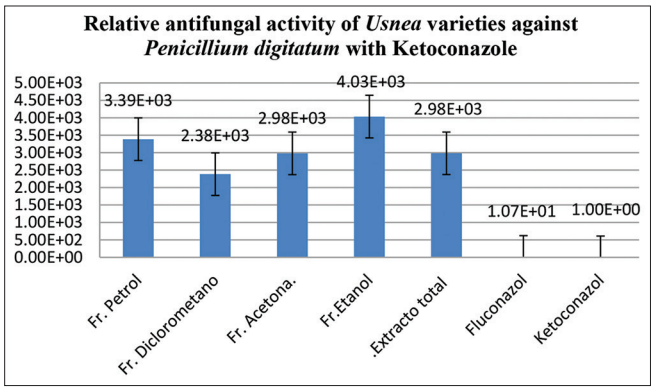

Fig. 3: *Penicillium digitatum* relative antifungal activity (AFR) with respect to ketoconazole, where when the activity of ketoconazole is 1 mg DMSO, the activity of each fraction and total extract of *Usnea* varieties, is greater

Figs. 4-6 describe antifungal activity of *Usnea* varieties against *R. nigricans* in Fig. 4 shows that the most favorable result is with petrol fraction (75.35 mm). Figs. 5 and 6 show that when the activity of fluconazole or ketoconazole is 1 mg the activity of each fraction and total extract of *Usnea* varieties is greater. The best relative antifungal activity was with diclorometano fraction.

Figs. 7-9 describe results with *A. niger*. Fig. 7 illustrates that the most favorable result is for total extract fraction (35.48 mm); it is the lowest

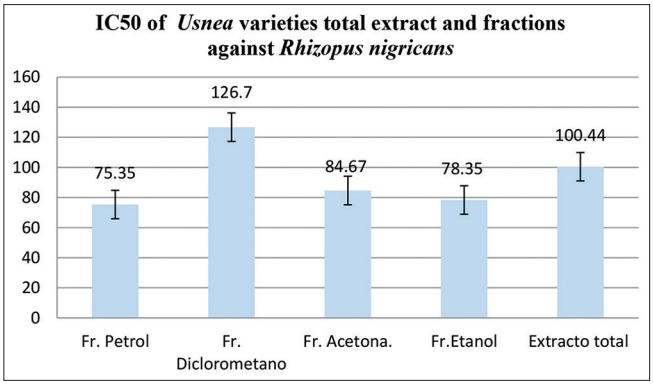

Fig. 4: IC_{50} results of *Rhizopus nigricans,* where the most favorable result is that the petrol fraction (75.35 mg) since it is the closest value to 50 and presents better activity

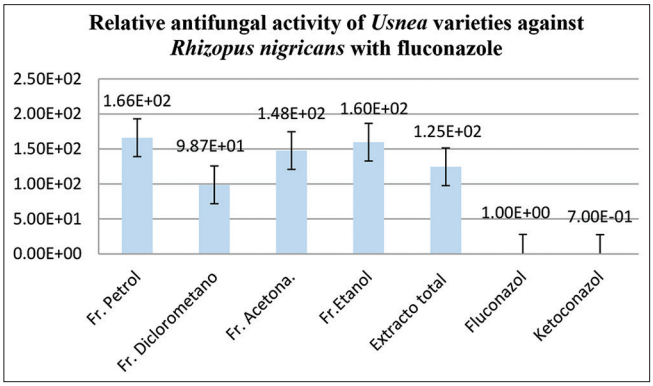

Fig. 5: *Rhizopus nigricans* relative antifungal activity (AFR) with respect to fluconazole, where when the activity of fluconazole is 1 mg DMSO, the activity of each fraction and total extract of *Usnea* varieties, is greater

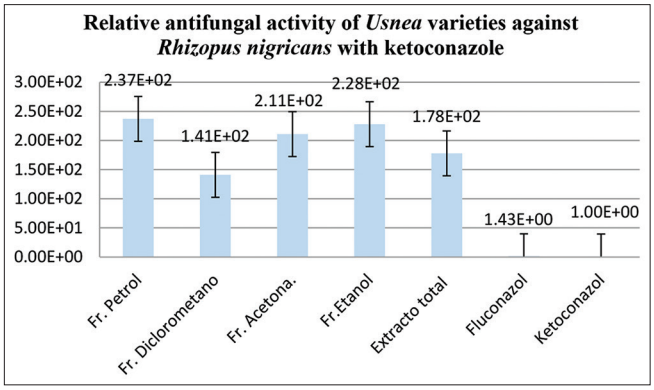

Fig. 6: *Rhizopus nigricans* relative antifungal activity (AFR) with respect to ketoconazole, where when the activity of ketoconazole is 1 mg DMSO, the activity of each fraction and total extract of *Usnea* varieties, is greater

value and presents better activity. In Figs. 8 and 9, we found relative antifungal activity (AFR) with respect to fluconazole and ketoconazole, where when the activity of fluconazole or ketoconazole is 1 mg, the activity of each fraction and total extract of *Usnea* varieties is bigger, the best activity extract was with dichloromethane, followed by petrol, acetone, and ethanol.

DISCUSSION

Medicinal herbs are essential of resource as it may contain active compounds which are able to be investigated in drug development [15].

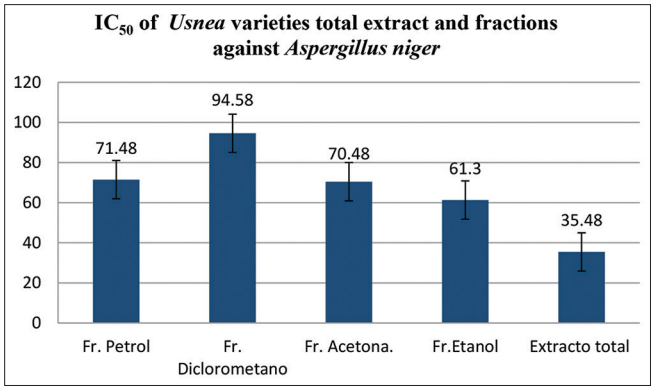

Fig. 7: IC_{50} *Aspergillus niger* where the most favorable result is for total extract fraction (35.48) is the lowest value and presents better activity

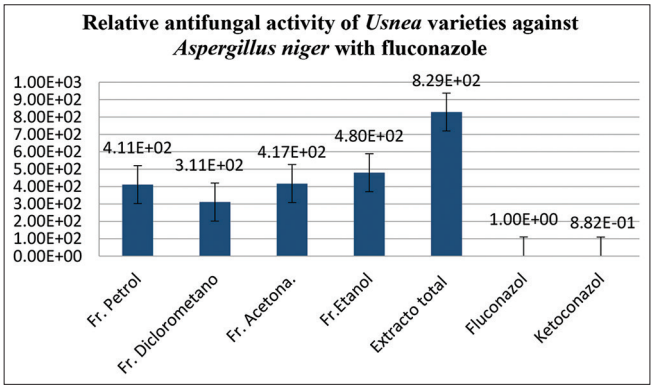

Fig. 8: *Aspergillus niger* relative antifungal activity (AFR) with respect to fluconazole, where when the activity of fluconazole is 1 mg DMSO, the activity of each fraction and total extract of *Usnea* varieties, is bigger

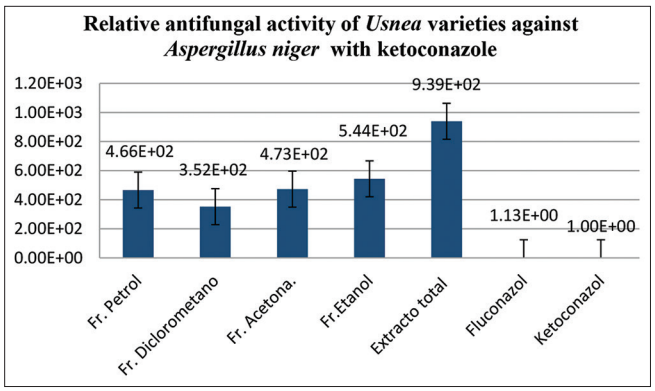

Fig. 9: *Aspergillus niger* relative antifungal activity (AFR) with respect to ketoconazole, where when the activity of ketoconazole is 1 mg DMSO, the activity of each fraction and total extract of *Usnea* varieties, is bigger

Lots of medicinal herbs which are used empirically in certain sites have been researched to analyze alternative agents for treating diseases such as fungal infection [16]. The result obtained from antifungal activity assay using different extracts showed that the growth of all fungi was inhibited. According to Kambizi and Afolayan [17], the percentages of inhibition by the lichen *Usnea barbata* with *A. niger* in concentrations of 10, 5, 1, and 0.5 mg were (61.57, 56.27, 27.80, 17.13, and 10.43, respectively), with an IC_{50} of 4.12. Values are not equivalent to our study. The extracts of different polarities of lichens are made up of a variety of substances in different proportions and diverse characteristics; it

is not possible to compare the "potency" with respect to the pattern. For this reason, it is worth purifying and identifying the extracts that showed activity against fungi, and thus verify the secondary metabolite or metabolites that presently said activity. *Usnea* varieties are promising since an extract or fraction that has many secondary metabolites have antifungal activities and therefore it is important to carry out other studies such as cytotoxic activity, antioxidant activity, anti-inflammatory activity, and antiparasitic activity to correlate them [18,19]. The strong presence of phenols, terpenes, tannins, and flavonoids in all the lichen samples tested supports lichen's antioxidant activity and conveys the embodiment of several important metabolites of pharmaceutical interest. Several reports have analyzed similar results, thereby grounding lichen compounds as an alternative drug source for treating diseases caused due to free radicals [20].

About comparing IC_{50} of varieties of *Usnea*, we found that the fraction with the best activity is acetone 58.53 mg/L for the petrol fraction, the IC_{50} had a value of 144.44 mg/L, dichloromethane fraction value of 114.43 mg/L the acetone fraction with a value of 58.53 mg/L, the ethanolic fraction value of 68.23 mg/L, and the total ethanolic extract with a value of 163.58 mg/L.

The fractions that showed the best activity were acetone and ethanolic fraction. We found that the fractions that had the best activity against *P. digitatum* were ethanolic fraction (44.33), for *R. nigricans* was petrol fraction (75.35) and for *A. niger* was the total extract (35.48). We found that antifungal relative activity to fluconazole and dichloromethane fraction has the best activity for all three fungi. Our results are in concordance with Ghate *et al*. [21] that found antifungal activity against *A. niger* and *P. digitatum,* low polarity fraction (petroleum ether) had the best results compared with azoles controls. For *A. niger*, they get an equivalent sensibility, 465.6 mg of the petroleum ether fraction are needed for each milligram of ketoconazole and 174.6 mg for each milligram of fluconazole.

To relate the standards with the extract and the fractions, the $1/IC_{50}$ was taken, thus obtaining the 1/AFR of each of them, thus the sensitivity obtained with 1 mg of fluconazole against *A. niger* is equivalent to 310.93 mg of the dichloromethane fraction. The sensitivity obtained with 1 mg of fluconazole against *P. digitatum* is equivalent to 222.15 mg of the dichloromethane fraction. The sensitivity obtained with 1 mg of fluconazole against *R. nigricans* is equivalent to 98.71 mg of the dichloromethane fraction.

For the relative antifungal activity with ketoconazole, the dichloromethane fraction also has the best activity; the sensitivity obtained from 1 mg ketoconazole for *A. niger* is equivalent to 352.39 mg, for *P. digitatum* it is equivalent to 2383.71 mg and finally in *R. nigricans,* it is equivalent to 140.97 mg of the fraction.

CONCLUSIONS

Extracts of different polarity obtained from *Usnea* varieties have inhibitory activity against the growth of the fungi *A. niger*, *P. digitatum,* and *R. nigricans.*

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AUTHORS' CONTRIBUTIONS

Luis Pombo, Oscar E Rodríguez design the experiments; Dayanna Cabra Gacha, Luis M. Pombo Ospina, Janeth Arias Palacios, and Oscar E. Rodríguez performed and analyzed the experiments and results. Janeth Arias and Oscar Rodríguez wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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