

Antifungal Activity of Leaves and Cones Extracts of *Pinus halepensis* in Tlemcen North-West of Algeria

Activités Antifongique des Extraits des Feuilles et des Cônes de *Pinus halepensis* de Tlemcen Nord-Ouest Algérien

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ABSTRACT

Background: plants represent an exhaustible source of chemical molecules with a diverse biological activity. In this context, our study deal with the antifungal effect of aqueous extracts of *Pinus halepensis* leaves and cones as well as bioactive substance (gallic acid) on pathogenic fungi namely: *Trichophyton rubrum*, *Microsporum canis*, and *Candida albicans*.

Methods: methods involving direct contact and diffusion on agar plates were used. Antifungal testing was performed using aqueous extracts

Results: the studied strains were sensitive to aqueous extracts of *P. halepensis* leaves and cones. The aqueous extract of *P. halepensis* leaves and cones of 200 mg/ml had a good antifungal activity as the percent inhibitions were respectively: 62% and 58% on *T. rubrum*, 80% and 65% on *M. canis*. The effect of the gallic acid is less significant in on both dermatophytes. The *C. albicans* strain was particularly sensitive to the extracts of *P. halepensis* leaves with inhibition areas of 18 mm diameter.

Conclusion: these results indicate that extracts of *P. halepensis* may constitute an alternative solution for treatment of mycosis dermatosis and using an in vivo study as a perspective

Keywords: *Pinus halepensis*, leaves, cones, antifungal activity, gallic acid.

RÉSUMÉ

Contexte : les plantes constituent une source inépuisable de molécules chimiques présentant une diversité d'activités biologiques. Dans ce contexte, notre étude porte sur l'effet antifongique des extraits aqueux des feuilles et des cônes de *Pinus halepensis*, ainsi que d'une substance bioactive (l'acide gallique), sur des champignons pathogènes : *Trichophyton rubrum*, *Microsporum canis* et *Candida albicans*.

Méthodes : les méthodes utilisées incluent le contact direct et la diffusion sur des boîtes de Petri gélosées. Des tests antifongiques ont été réalisés à partir d'extraits aqueux

Résultats : les souches étudiées se sont révélées sensibles aux extraits aqueux des feuilles et des cônes de *P. halepensis*. L'extrait aqueux des feuilles et des cônes de *P. halepensis* à 200 mg/ml a montré une activité fongicide notable, avec des pourcentages d'inhibition respectifs de 62 % et 58 % sur *T. rubrum*, et de 80 % et 65 % sur *M. canis*. L'effet de l'acide gallique s'est avéré moins significatif sur les deux dermatophytes. La souche *C. albicans* s'est particulièrement montrée sensible aux extraits de feuilles de *P. halepensis*, avec des zones d'inhibition atteignant 18 mm de diamètre.

Conclusion : Ces résultats suggèrent que les extraits de *P. halepensis* pourraient constituer une solution alternative dans le traitement des dermatoses mycosiques et utiliser une étude in vivo comme perspective

Mots-clés: *Pinus halepensis*, feuilles, cônes, activité antifongique, acide gallique.

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INTRODUCTION

Dermatophytes are a group of closely related filamentous fungi that invade keratinized humans and animal's tissues such as skin, hair and nails causing dermatophytosis. They are an important cause of superficial fungal infection [1, 2]. In medical mycology, there are filamentous fungus including dermatophytes which are classified in three genera, Epidermophyton, Microsporum, and Trichophyton as well as Zygomycetes, molds and yeast [3]. Individuals are at high risk contamination with a particularly harsh and constraining treatment [4].

Nowadays, extracts of plants and compounds derived from plants, considered as the backbone of traditional medicine, are used in the prevention and the treatment of a wide range of diseases including mycoses [5]. Furthermore, these medicinal plants are a major source of bioactive molecules which can be valuable in the formulation of new anti-fungal agents [6].

Few studies have investigated the antifungal activity of *P. halepensis* leaves and cones. This study aims to isolate and identify dermatophytes from patients at the University Hospital Center (UHC) of Tlemcen, and to evaluate the antifungal potential of *P. halepensis* extracts from the Tlemcen region (Algeria) against the isolated fungi and a *Candida* strain.

METHODS

Isolation of dermatophytes

Collection

The samples were collected at the dermatology ward of Tlemcen in patients affected by the fungus. Sufficient and Adequate samples were taken to isolate the pathogenic agent in patients without any antifungal therapy. According to the type of lesion, sampling was conducted as follows. Scales were collected and hairs (healthy and broken) were pulled UHC out with sterile epilator forceps and kept in a sterile Petri dish. For alopecia areata, lesions were collected separately. Skin scales and crusts were collected by scraping across the margin of the lesions using a sterile scrapper and kept in a sterile Petri dish. For inflamed lesions, a swab humidified with sterile water was applied on the lesion. After using a disinfectant, the nails involved were cut to the limit of the tissues using sterile scissors.

Direct microscopic examination

The microscopic examination highlighted mycelia branching and confirmed the clinical diagnosis of dermatophytes. Few drops of potash were spread on the collected sample which was placed in a blade and a slide and heated for some seconds. Potash was used to soften keratin. After one hour, the blades were examined under the microscope to observe mycelia branching and spores to confirm the presence of fungi.

Culture

After direct microscopic examination, positive samples were inoculated using a platinum loop or a sterile forceps

in a Sabouraud base supplemented with chloramphenicol and/or actidione which inhibits bacterial growth. The boxes were incubated at 25 °C for 10 days up to 6 weeks. Cultures had to be examined twice a week to follow the colonies changing aspect and the growing duration.

Purification

Purification was done with successive sub culturing on a Sabouraud base or a growth media mainly Potatoes Dextrose Agar (PDA) regarding *M. canis* or agar with urea for *T. rubrum*. Fragments or disks containing dermatophytes culture were placed in the medium using a platinum loop or a Pasteur pipette.

Identification of dermatophytes

Macroscopic morphology of cultures

This examination consisted of observing with the naked eye the colonies obtained from the Sabouraud base by taking into account the following criteria: size, colour (both sides), shape, surface characteristics and the presence of a diffused pigment for agar.

Microscopic morphology of cultures

The microscopic identification consisted of depositing fragments of cultures and methylene blue stain on a blade and a slide to be examined under the microscope. An adhesive tape may be carefully applied on the colonies. The microscopic examination highlighted mycelia branching, the diameter, the morphology, conidia aspect (macro or micro conidia) and the existence of organs of fructification. Dermatophytes identification was done according to the manual under the heading "Examen direct et place de l'histologie en mycologie" [7].

Antifungal activity testing

Antifungal testing was performed using aqueous extracts of *P. halepensis* leaves and cones as well as a bioactive substance (gallic acid). These extracts were provided by the Laboratory for antibiotic, antifungal, physiochemical synthesis and biological activity testing. Two in vitro techniques were performed, agar diffusion method (agar well diffusion method) and direct contact method. Two types of strains were isolated, *M. canis* and *T. rubrum* (dermatophytes) collected from Tlemcen UHC and *C. albicans* (yeast) from Laboratory LABSAP University of Tlemcen.

Preparation of extractive solutions

A quantity of 0.03g of aqueous extract of *P. halepensis* leaves and cones were mixed with 3 ml of distilled water to obtain a stock solution of 10mg/ml. Extractions were carried out with the following method: in a round bottomed flask connected to a condenser, 5g sampling extracted under reflux using distilled water, ethanol and diethyl ether. Crude extracts were filtered through a rotavapor and stocked at 4°C for further use [8]. All analyses were conducted in triplicate and results were based on dry weight per 100 grams of sample.

C. albicans inoculum preparation

The strain was grown on Sabouraud dextrose agar. Following incubation at 30°C for 24 hours, colonies were transferred in a nutrient broth or in physiological water. After incubation at 25°C for 24 hours, the optical density of a 0.5 McFarland standard was measured with a spectrophotometer, the suspension had to contain 106 CFU (colony forming unit)/ml [9].

Preparation of dermatophyte spore's suspension

The production of a suspension of spores adequate for use was obtained after scrapping mycelia cultures from a fungal strain developed in a PDA medium grown for 15 days. The resulting mycelium was mixed with sterile water. Then, the mixture was filtered on Watman paper N°1. The suspension density was adjusted at 107spores/ml after evaluating the initial density of Malassez cell [10].

Activity of C. albicans extract

Using a pipette Pasteur, a round circle is cut on a Sabouraud agar grown previously with a C. albicans suspension. Then, 50 µl of the extract is put with a micropipette on different concentrations areas (100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml). The boxes are incubated at 25°C for 24 hours. The diameter of inhibition areas is measured following extracts diffusion in the agar [11].

Activity of dermatophyte extract

A volume of 100 µl from different extract concentrations was added to 20 ml Sabouraud base undercooling in a test tube. The mixture was subject to vortex shaking and poured in Petri dishes. Inoculation was performed with a disk of 6 mm diameter placed at the center of the dish precultured. A Petri dish containing 20 ml of Sabouraud Base with no extract was seeded with a fungus disk and used as a control strain. Incubation was performed at 25°C for 1-3 weeks according to species. The antifungal activity was assessed by measuring the diameter growth of dermatophytes in presence of extract [12]. After incubation, we evaluated the inhibition rate by using Wang formula [13].

Statistical study

The data was analyzed using SPSS software. Statistical Package for the Social Sciences for Windows (version 25.0 ; Armonk, NY : IBM Corp., USA). It should be noted that the effects on the diameters of the zones of inhibition are statistically significant, as their significance levels are $p < 0.05$.

RESULTS

Isolation of Dermatophytes

Sampling

In order to isolate fungi from the skin, samples were collected from patients at the Dermatology Department of Tlemcen UHC. The results of examinations are presented in Table 1.

Table 1. Number of samples collected at Tlemcen UHC.

Number of samples	Sample quality	Sample positivity
E1	scalp	Microscopic moth
E2	Right foot nail	DPH -
E3	scalp	Microscopic moth
E4	scalp	Microscopic moth
E5	scalp	moth
E6	Right foot nail	DPH+
E7	Squamous thigh buttock	DPH+
E8	Right foot squamous	DPH+
E9	Scalp	DPH+
E10	Right foot nail	DPH+
E11	Kerion	DPH-
E12	Right foot squamous	DPH-
E13	Left foot nail	DPH-
E14	Left foot nail	DPH-
E15	Right foot squamous	DPH-
E16	Right foot nail	DPH-
E17	Right foot nail	DPH-
E18	Right foot nail	DPH-
E19	Scales on the right foot	DPH-
E20	Left foot nail	DPH-
E21	Ongle pied droit	DPH-

*DPH + : positive sample presence of mycelia branching of dermatophytes,

†DPH - : absence of mycelia branching of dermatophytes.

Phenotypic identification of dermatophytes strains

The study involved macroscopic and microscopic features, according to the results of macroscopic and microscopic identification referring to Chabasse's guide [14], the two isolates strains of Tlemcen UHC belonged to two species *T. rubrum* and *M. canis*. According to findings, *T. rubrum* was isolated from toes and *M. canis* was isolated from scalp. After 10 days of incubation, the *M. canis* culture appeared as a white, small, star-shaped surface with a yellow-orange reverse pigment. This is shown in Figure 1.



Figure 1. Photographs of *Microsporium canis* colonies recto A verso B.

The *T. rubrum* culture, after 8-10 days of growth, developed a curved, white, and densely cottony surface. Its reverse pigment was brown, as shown in Figure 2.

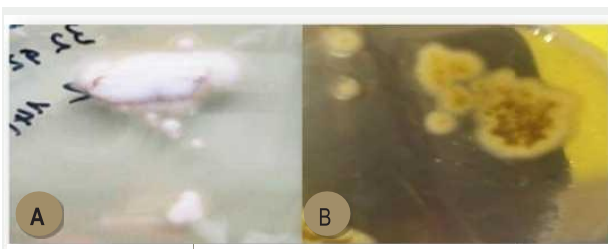


Figure 2. Photographs of *Trichophyton rubrum* colonies (A- recto and B-verso).

The microscopic aspect of *M. canis* revealed thin, branching mycelia and spindle-shaped macronidia containing 6-10 echinulate cells (Figure 3).

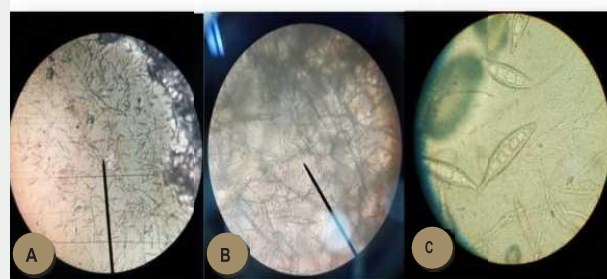


Figure 3. Photographs of the microscopic appearance of *M. canis* A-G x 10, B-G x 40, C-G x 400.

The microscopic examination of *T. rubrum* revealed a branching mycelium. Pyriform, teardrop-shaped micronidia were observed, along with a small number of pencil-shaped macroconidia, which were almost absent in some cases (Figure 4).

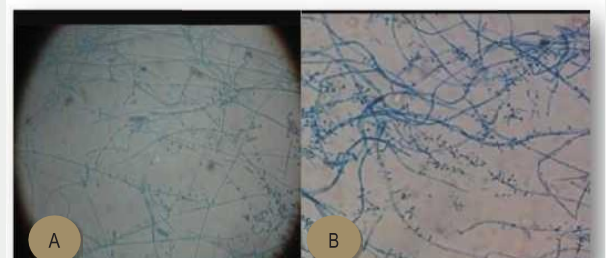


Figure 4. Photographs of the microscopic appearance of *T. rubrum* (A- G x100, B-G x 400).

Antifungal activity testing

Aqueous extracts and gallic acid effects on *C. albicans*

Different concentrations of prepared extracts were tested on *C. albicans* strain and in order to determine the antifungal activity, the size of the zones of inhibition around the disks was measured and recorded. The presence or the absence of a halo indicates the criteria of sensibility towards the used extracts. According to figure 8.400 µg/ml of gallic acid revealed a good inhibition of *C. albicans* (diameter = 19 mm) followed by extracts of *P. halepensis* leaves and cones being respectively: 18 mm and 16 mm (Figure 5).

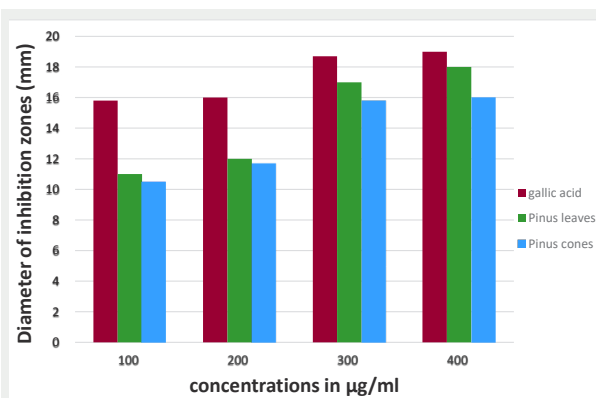


Figure 5. Diameter of *C. albicans* inhibition zones as a function of extract concentrations (well method).

Aqueous extracts and gallic acid effects dermatophytes

In order to determine the antifungal activity, the growth zone diameter of the strains was measured in presence of the tested extracts.

According to findings, extracts of *P. halepensis* cones and leaves had a good antifungal activity against *T. rubrum*. The percent inhibition was respectively of 62% and 58% (Figure 6). These molecules inhibited *T. rubrum* and reduced the mycelia growth as compared to the control strain.

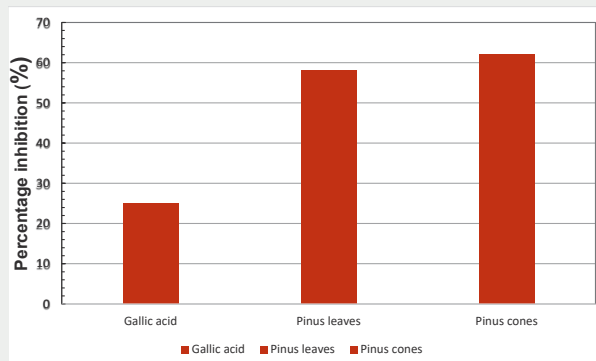


Figure 6. Percentage inhibition of *T. rubrum* by the different extracts used.

These molecules inhibited *T. rubrum* and reduced the mycelia growth as compared to the control strain. The best percent inhibition was recorded for extracts of *P. halepensis* cones with 80% followed by extracts of *P. halepensis* leaves with 65 % (Figure 7).

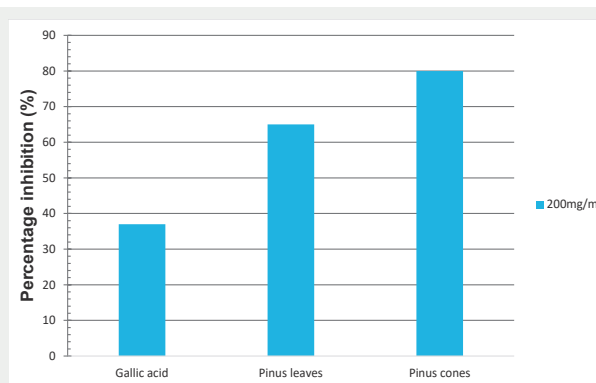


Figure 7. Inhibition of *M. canis* by the different extracts used.

Statistical study

The method used for data analysis is the two-factor ANOVA with factors "Product" and "Concentration." The objective is to determine whether each of the two factors and their interaction are statistically significant. The table 2 and 3 presents the mean and standard deviation of the diameters of inhibition zones for each combination of the factors "Product" and "Concentration," as well as for the "Total" rows.

Table 2. Descriptive statistics Dependent variable : diameters of inhibition zones (mm)

Product	Concentration (µg/ml)	Mean diameter	Standard deviation	N
Gallic acid	100	15.8000	1.01489	3
	200	16.0000	.62450	3
	300	18.7000	.36056	3
	400	19.0000	1.27671	3
	Total	17.3750	1.72317	12
Leaves of <i>P. halepensis</i>	100	11.0000	1.37477	3
	200	12.0000	.26458	3
	300	17.0000	.62450	3
	400	18.0000	.26458	3
	Total	14.5000	3.24514	12
Cones of <i>P. halepensis</i>	100	10.5000	1.32288	3
	200	11.7000	1.15326	3
	300	15.8000	2.26495	3
	400	16.0000	.36056	3
	Total	13.5000	2.82875	12
Total	100	12.4333	2.75500	9
	200	13.2333	2.18403	9
	300	17.1667	1.73349	9
	400	17.6667	1.48577	9
	Total	15.1250	3.08752	36

Table 3. Descriptive statistics (continued) Dependent variable : diameters of inhibition zones (mm).

Product	Concentration (µg/ml)	Mean	Standard error	Confidence interval (95 %)	
				Lower limit	Upper limit
Gallic acid	100	15.800	.622	14.516	17.084
	200	16.000	.622	14.716	17.284
	300	18.700	.622	17.416	19.984
	400	19.000	.622	17.716	20.284
Leaves of <i>P. halepensis</i>	100	11.000	.622	9.716	12.284
	200	12.000	.622	10.716	13.284
	300	17.000	.622	15.716	18.284
	400	18.000	.622	16.716	19.284
Cones of <i>P. halepensis</i>	100	10.500	.622	9.216	11.784
	200	11.700	.622	10.416	12.984
	300	15.800	.622	14.516	17.084
	400	16.000	.622	14.716	17.284

The figure 8 illustrates the mean diameters of the inhibition zones for each combination of the factors "Product" and "Concentration." It suggests the absence of interaction between the two factors, as the lines appear parallel (no crossing).

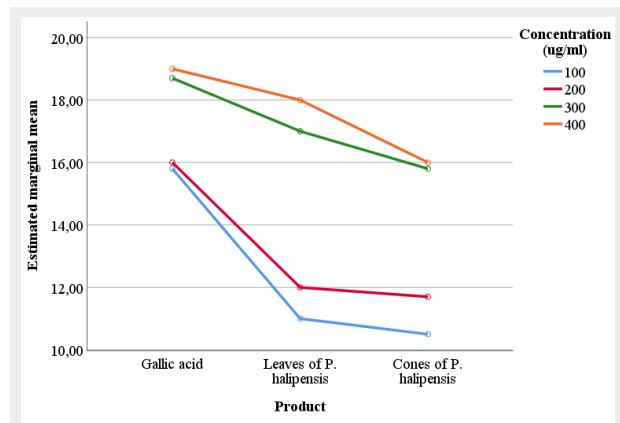


Figure 8. Estimated marginal means of diameters of inhibition zone.

The ANOVA table is presented in table 4. The model fit for the ANOVA data is of high quality, as the adjusted coefficient of determination (R^2) is close to 1. The particular lines of interest are those for "Product," "Concentration," and "Product × Concentration." There is no statistically significant interaction between the two factors "Product" and "Concentration," as the p-value for the "Product × Concentration" line is 0.075. The effects of each of the two factors were interpreted independently. The lines for "Product" and "Concentration" indicate that these factors have a statistically significant effect on the inhibition zone diameters, as their significance levels are $p < 0.05$.

Table 4. Tests of between-subjects effects Dependent variable : diameters of inhibition zones (mm).

Source	Sum of type III squares	df	Mean square	F	Significance
Model	8541.330 ^a	12	711.778	612.721	$< 10^{-4}$
Product	97.125	2	48.563	41.804	$< 10^{-4}$
Concentration	193.067	3	64.356	55.400	$< 10^{-4}$
Product * Concentration	15.575	6	2.596	2.235	.075
Error	27.880	24	1.162		
Total	8569.210	36			

a. R-squared = .997 (Adjusted R-squared = .995).

All three products differ significantly in terms of inhibition zone diameters. Post-hoc tests, such as Fisher's LSD (Least Significant Difference), can be used for pairwise comparisons among products. The table 5 summarizes these comparisons : the differences in inhibition zone diameters are statistically significant between gallic acid and leaves of *P. halepensis* (row 1), leaves and cones of *P. halepensis* (row 2), and cones of *P. halepensis* and gallic acid (row 3), as the significance levels of each comparison are $p < 0.05$.

DISCUSSION

According to isolation results, *T. rubrum* was isolated from toes and *M. canis* was isolated from scalp as well as tinea. The last specie is the main cause of scalp affections whereas, *T. rubrum* is usually the main cause of onychomycosis [1, 2].

Table 5. Multiple comparisons Dependent variable : diameters of inhibition zones (mm).

(I) Product	(J) Product	Mean difference (I-J)	Standard error	Significance	Confidence interval (95 %)	
					Lower limit	Upper limit
Gallic acid	Leaves of <i>Pinus</i>	2.8750*	.44001	< 10 ⁻⁴	1.9669	3.7831
	Cones of <i>Pinus</i>	3.8750*	.44001	< 10 ⁻⁴	2.9669	4.7831
Leaves of <i>P. halepensis</i>	Gallic acid	-2.8750*	.44001	< 10 ⁻⁴	-3.7831	-1.9669
	Cones of <i>Pinus</i>	1.0000*	.44001	.032	.0919	1.9081
Cones of <i>P. halepensis</i>	Gallic acid	-3.8750*	.44001	< 10 ⁻⁴	-4.7831	-2.9669
	Leaves of <i>Pinus</i>	-1.0000*	.44001	.032	-1.9081	-.0919

Calculation based on observed means : the mean difference is *Significant at the p < 0.05 level.

The antifungal activity of aqueous extracts of *P. halepensis* cones and leaves and gallic acid effects on *C. albicans* against the three species *C. albicans*, *M. canis* and *T. rubrum* have revealed interesting results. *C. albicans* strain was sensitive to *P. halepensis* cones and leaves via the agar well diffusion method with zones inhibition of 17 mm and 15.8 mm at 300 µg/ml and about 18 mm and 16 mm at 400 µg/ml. Many studies [15-16] have revealed that aqueous extracts of plants are effective against fungi. Antimicrobial effects of *P. halepensis* Mill., were attributed to different chemical classes such as terpenoids, phenolic acids, flavonoids, fatty acids and steroids, aldehydes and ketones [17]. Aqueous extracts of many plant species have inhibited the growth of *Candida* species including *C. albicans* [16].

Antimicrobial activity of *P. halepensis* extracts is related to the presence of tannins linked to the wall proteins which modify the membrane permeability and allow moving in the fungal cell with cytotoxic activity implying antifungal activity [18]. *P. halepensis* is known for its effective components like terebenthine essential oil found to possess interesting antifungal and antimicrobial properties [17].

Our findings indicate that the collected extracts of *P. halepensis* cones and leaves have been active against dermatophytes species such as: *T. rubrum* and *M. canis* with percent of inhibition 58 and 62% against *T. rubrum* as well as interesting results of cones extracts against *M. canis* (65 % and 80 %). Scientific literature indicates that aqueous extracts of plants might be effective against filamentous fungi like *Fusarium* and *Phytophthora* [19] and more specifically against dermatophytes including *Trichophyton* [17]. The inhibition could be explained by the presence of phenolic components in the aqueous extract which can act on disruption of membranes, inhibition of cell wall synthesis, hyphal growth, and spore germination [20].

Aleppo Pine is known for its antifungal properties, *P. halepensis* tar was more inhibiting of the mycelium growth of many fungi including *Fusarium solani*, *Fusarium nyagami* with an inhibition rate of 77%. [21]. The inhibition zone on *C. albicans* with gallic acid is 19 mm at 300 µg/ml using the agar well diffusion method. On the other hand, this compound has shown slight inhibition on *T. rubrum* (30%) unlike *M. canis* which proved to be resistant.

The absence of inhibition does not necessarily imply that the sample is not active. Some products are slow to diffuse in culture medium [22].

According to [23], gallic acid have an interesting antifungal activity leading to disruption of membrane integrity and

alteration of mitochondrial transmembrane potential.

CONCLUSION

P. halepensis cones and leaves demonstrate noteworthy antifungal activity against *Candida albicans* with major inhibition percent observed on *M. canis* and *T. rubrum*. These results are promising and indicate that *P. halepensis* extracts can be used to treat skin mycoses. A detailed phytochemical characterization of these extracts contributes to identify the components responsible for this inhibition.

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