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Antifungal Activity of Topical Formulation Containing *Artemisia Herba Alba Asso* Essential Oil

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Abstract

Given the increase in antibiotic resistance phenomena and the undesirable effects of synthetic drugs. Medicinal plants are used as a direct or indirect source of the active ingredients. Our research constitutes a development of essential oils from *Artemisia herba alba Asso* cultivated in the Mascara region. A hydrophobic ointment has been formulated based on this essential oil and tested against five strains of *Candida albicans*. Several physicochemical and microbiological tests were used to verify the quality and the toxicity of the product. Then, the determination of the antifungal activity of this preparation was assessed against five strains of *Candida albicans* (S1, S2, S3, S4 and S5) by the disk-diffusion agar method. The results revealed that this preparation was devoid of total aerobic germs, yeasts and molds. However, the pH value was found equal to 5.98. In addition, the irritation primary index was marked less than 0.5. The ointment was powerful against S1, S3, S4 and S5 strains with inhibition diameters ranging from 16± 4 mm to 23 ± 2 mm. The Nystatin ointment was observed active against strains S2, S4 and S5 with diameters of the zones of inhibition; 21 ± 2 mm, 21± 1 mm and 20 ± 3 mm respectively. The ointment formulated with essential oil from *Artemisia herba alba asso* has proven useful against candidiasis caused by *Candida albicans* species.

1. Introduction

One of the major originalities of plants is their ability to accumulate frequently secondary metabolites, which represent an important source of molecules usable by humans in fields as different as pharmacology or the food industry **(Macheix *et al.*, 2005).** *Artemisia herba alba*, or known as sagebrush, is a plant characteristic of the Middle East, from North Africa, Southern Europe to the Himalayan mountains, it is used in herbal medicine to treat several diseases. Several structural types of sesquiterpene lactones have been found in the essential oils of the aerial parts of *A, herba alba*. The eudesmanolides followed by the germacranolides **(Bouldjadj, 2009).** The skin infection is usually treated with topical amphotericin, clotrimazole or nystatin **(Brown *et al.*, 2012).** The success of topical antifungal is attributed to its mode of action, the fungistatic doses and that they are not toxic on other host cells **(Martini, 2011).** Therefore, our choice fell on the formulation of a hydrophobic ointment based on the use of essential oils as an active ingredient. Then, its antifungal activity was carried out against five strains of *Candida albicans* by the diffusion method on agar.

2. Material and methods

2.1 Chemical products

Paraffin oil (Pharma services), glycerol (Pharma services), sodium benzoate (APAC), petroleum jelly (Phyto tech), dimethyl sulfoxide (Thermo Scientific), sterile tween 80 (Proteomic Grade), buffered sodium chloride-peptone (HIMEDIA).

2.2 Cultures

Cellulose filter membrane, 0.45 µm (Rotilabo), TSA medium (LABKEM), Sabouraud medium (Bio-Rad), nutritive broth (Bio-Rad), chloramphenicol-actidione medium (Bio-Rad), sterile physiological water, mueller Hinton agar (Bio-Rad).

2.3 Equipment

Mortar, pH meter (Radiometer), water bath (Memmert).

2.4 *Artemisia herba alba Asso* essential oil

Artemisia herba alba Asso was harvested in October 2015 in Mascara region and stored as voucher specimen in the herbarium of the university of Mascara under the code (AS00006). The extraction of essential oils and their compositions revealed by the GC / MS were described previously **(Boukhennoufa *et al.*, 2019).**

2.5 Animals

The rats belong to the *Rattus norvegicus* species were supplied by the pet store at the University of Mascara. However, the animals were acclimated to laboratory conditions at least five

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days before the experiment. The breeding was carried out according to the guide for the use and care of laboratory animals **(National Institutes of Health, 2011).**

2.6 Fungal strains

Five strains of *Candida albicans* were used for this study. The isolation and the identification of these microorganisms were described previously **(Boukhennoufa *et al*., 2019).**

2.7 Preparation of the ointment

The ointment is a hydrophobic type (Table 1) modified and adopted previously **(Biyiti *et al.,* 2012).** In a blender, the essential oil of *Artemisia herba alba asso*, paraffin oil and glycerol were introduced. The whole was brought to mixing for 10 min. Then, the sodium benzoate was added and mixed again for 10 min. The initial mixture recovered was placed in a mortar. The petroleum jelly was then added to the preceding mixture little by little by grinding until obtaining a creamy consistency and fine to the touch. The ointment was kept at 5°C in a dry place until use.

Table 1. Preparation of an ointment based on *Artemisia herba alba asso* essential oil (10%)

Constituants	Role	Quantity (g)
Artemisia herba alba asso essential oil	Active ingredient	10
Paraffin oil	Excipient	1.83
Glycerol	Excipient	10
Vaseline	Excipient	76.68
Tween 80	Emulsifier	0.49
Sodium benzoate	Conservative	1

2.8 Checking the quality of the ointment

2.8.1 Macroscopic characters
The macroscopic nature of the ointment was evaluated by observing the color, consistency and odor **(Sanogo *et al*., 2006).**

2.8.2 Homogeneity
The homogeneity of the ointments was checked by spreading a thin layer on a flat surface using a spatula. The regular distribution or not of the extracts in the excipients has been noted **(Sanogo *et al*., 2006).**

2.8.3 pH measurement
The pH value was determined by measuring a dilution to one tenth of each ointment in hot distilled water. Under the same conditions, the pH of each excipient was measured **(Sanogo *et al*., 2006).**

2.8.4 Microbiological quality
The ointments formulated have undergone microbiological quality control. The purpose of this test is to count total aerobic flora mesphile (TAFM), yeasts and molds. According to the monographs of the European Pharmacopoeia, the load of TAFM, yeasts and molds counted in a dispensing preparation will not exceed 100 CFU/g (**Pharmacopée européenne, 2002).**

2.9 Sample preparation

Ten grams of ointment was mixed with 2 g of sterile tween 80. Then, the mixture was introduced into a water bath set at 40°C. In order, to obtain a homogeneous mixture during handling. After homogenization, a volume of 90 ml of buffered sodium chloride-peptone (pH 7.0) was added to the preceding mixture. In order, to obtain a dilution of one in ten of the original product **(Pharmacopée européenne, 2002).**

2.10 Enumeration of germs

This protocol was applied according to the monographs of the European Pharmacopoeia (2002). The prepared sample (100 ml) was then passed through a cellulose filter membrane having a pore size of not more than 0.45 µm. The membranes were then transferred to the surface of the culture medium (TSA) for the enumeration of total viable microorganisms. While yeasts and molds were counted after the transfer of the membrane to the surface of the Sabouraud medium. Then, the incubation was done from 30 to 35 ° C for 5 days for bacteria and from 20 to 25°C for the demonstration of the presence of yeasts and molds **(Pharmacopée européenne, 2002).**

2.11 Determination of the primary irritation index (PII**)**

As part of the study of the antifungal activity of the prepared ointment, a determination of the primary irritation index was necessary in order to assess the degree of toxicity after short-term dermal exposure. This test was carried out **(Derelanko *et al*., 1993)**, where rabbits were substituted by rats. Because, of their availability and their physiology close to that of humans. The back of each animal was divided into two areas. One scarified and the other remains intact. Then, a weight of 0.5 g of the product was applied to the two parts and kept in contact with the skin by a dressing and an adhesive tape (Fig 1). Then the observations were made one hour, 24 and 72 hours after the application of the product. The PII value was calculated by an irritation and edema rating system. In order to bring out the average PI and to deduce the irritant effect of the product applied. The primary irritation index was determined by the following equation: **PI = (Edema + Erythema) Treated flank + (Edema + Erythema) Control flank / 24**.

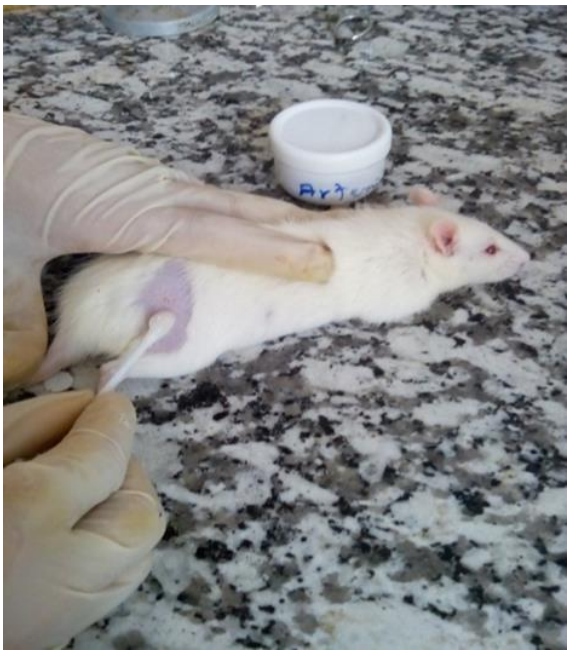

Figure 1. Ointment applying on the rat's skin to determine the primary irritation index

2.12 Antifungal activity of the ointment

The study of antifungal activity was carried out in a similar manner to that of an aromatogram. The reactivation was carried out in a liquid medium, nutritive broth for 24 h at 30°C **(Chopinet *et al.*, 2013**). Then a second subculture was carried out on Sabouraud-Chloramphenicol-actidione medium, incubated at 37 °C for 24 hours. From young cultures, three clones were inoculated into 5 ml of sterile physiological water. The suspension was standardized at the rate of 2 MC Farland **(Mighri *et al.*, 2010)**. 500 mg of the ointment were mixed with 1 ml of *dimethyl sulfoxide* (DMSO). At the same time, the Nystatin reference ointment was used as a positive control. However, the placebo was considered as a negative control. Petri dishes poured through the Mueller Hinton (MH) medium were inoculated beforehand with the strains (S1, S2, S3, S4 and S5) of *Candida albicans* by swabbing. The discs were impregnated with a volume of 10 µl of each solution and deposited on the surface. Then, the dishes were put at diffusion at 4 ° C for 30 min. Then, incubated for 48 h at 37°C. The effectiveness of the ointments against *Candida albicans* strains were expressed by measuring the diameter of inhibition all around the discs **(Ponce *et al.*, 2003).**

2.13 Statistical analysis of data

The experimental results were expressed as mean ± standard deviation (SD) of multiple replicates. Then, ANOVA (one way) was used to treat results, followed by Bonferroni's multiple comparison. The P values found below to 0.05 were considered statistically significant.

2.14 Ethics Statement

This study protocol was approved by the Local Ethical Comity of the University, based on adequately performed laboratory and animal experimentation according to the Helsinki Declaration (1964).

3. Results

3.1 Verification of the quality of the ointment

Drugs administered by the dermal route were much more effective and pose fewer problems than those of the other routes. This is why we choose the most classic formulation, the ointment. The quality of the ointment was assessed following numerous physical and microbiological tests (Fig 2). The formulated ointment was white in color, very smooth to the touch and easy to spread, with a characteristic smell of *Artemisia herba alba Asso.* The pH value was found equal to 5.98, this prevents the growth of germs promoting the degradation of its microbiological and organoleptic quality. On the other hand, no TAMF, yeasts and molds were detected on these products, in order to confirm their harmlessness before the determination of the antifungal activity *in vitro* against *Candida albicans.*

3.2 Determination of the primary irritation index (PII) of the formulated ointment

The PII determination results were determined using the Draize scores that appeared after 24 h and 72 h of application of the product. A value of 1 of erythema and edema of the sacrificial area were observed in the sixth rat after 24 h of skin contact with the product. These signs were completely gone after 24 h. While, the PII was found equal to 0.08. Since this value was strictly less than 0.5. Therefore, this ointment was qualified as non-irritating.

Figure 2. The formulated ointment

3.3 Study of the antifungal activity

A significant effect of the ointment was found against S1, S3, S4 and S5 strains with inhibition diameters ranging from 16 ± 4 mm to 23 ± 2 mm Compared to the placebo formulation with p value equal to 0.004. No significant effect of *Artemisia* ointment was observed compared to that of reference, nystatin against the tested strains (P= 0.66). However, the S2 strain was marked resistant to this ointment (Fig 3 and 4).

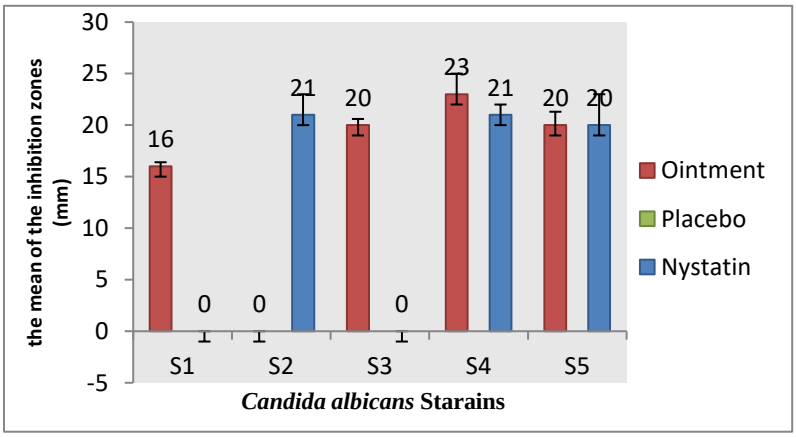

Figure 3. The average diameter of the inhibition zones, expressed in mm of the ointment, placebo and nystatin.

The negative control (placebo) did not induce any fungal inhibition. In addition, the reference ointment (Nystatin) was active against strains S2, S4 and S5 with diameters of the zones of inhibition: 21 ± 2 mm, 21± 1 mm and 20 ± 3 mm respectively. This difference in sensitivity against the fungal strains tested can be attributed to the quantity and quality of the active chemical compounds appreciated in essential oils.

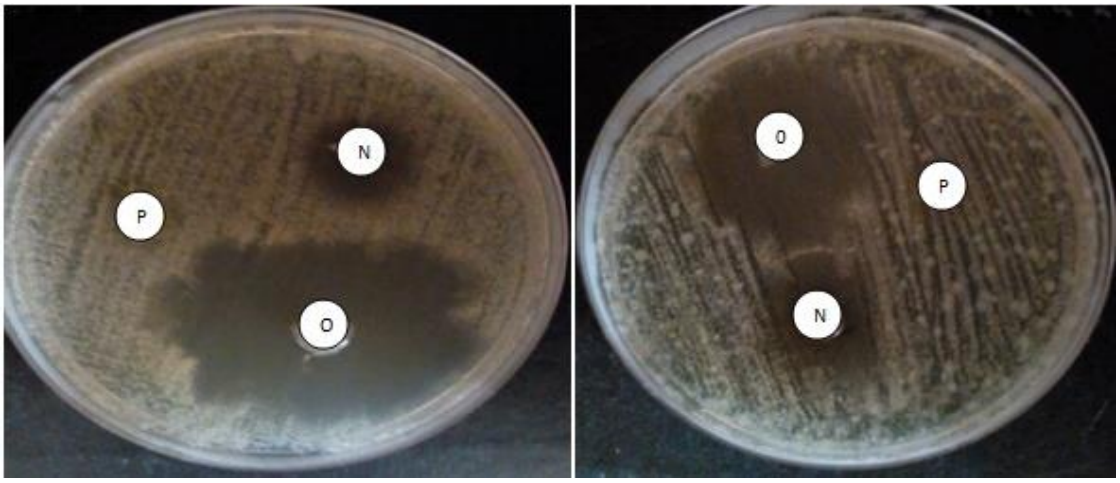

Figure 4. Inhibition zones of formulated ointment (O), placebo (P) and Nystatin (N). a: against the S3strain. b: against the S4 strain.

4. Discussion

The present study was conducted to determine the efficacy of an ointment contained *Artemisia herba alba asso* essential oil, used in the traditional pharmacopoeia of Mascara region. It should be noted that all the phenomena of erythema and edema were marked at the level of the scarified flanks. This is can be linked to the direct penetration of the ointment into the dermis. Because the epidermis layer is already damaged by scarifications. Any skin that is sick injured or modified by inflammation has an increased permeability of dermatological preparations compared to intact skin. And its risk must be taken into account **(Vandamme *et al.*, 2010).** By comparison with previous works, our results were similar to those reported, where she obtained a PII = 0.7 of an anti-cold ointment prepared with the essential oil of *Laurus nobilis* **(Guedouari, 2012).** The irritant power of a cream prepared from *Moringa oleifera* leaf extract was evaluated according to a single application by the 48-hour semi-occlusive patch test. However, no erythema or edema was observed after application **(Ali *et al.*, 2013).** In addition, the primary irritation index is equal to zero of an antibacterial soap prepared with oils from medicinal plants from the African pharmacopoeia, *Mareyami crantha, Mitracarpus scaberet Cassia alata* **(Soumahoro *et al.*, 2016).** In addition, the different effects of a single essential oil on the different fungal strains may be due to the variation in the genotype, although the five strains belong to the same species of *Candida albicans*. The antifungal activity of these essential oils can be attributed to its richness in oxygenated monoterpenes (80.85%) and in hydrocarbon monoterpenes (11.4%) already detailed previously **(Boukhennoufa *et al.*, 2019)**. The effect of the different doses of essential oil of *Artemisia herba alba asso* on the fungal strains, namely 0.05% and 0.25% indicate the mycelial decay of *Stemphylium solani*, *Fusarium moniliforme, Fusarium solani* and *Fusarium oxysporum* tested. While, the concentration of 0.75% completely prevented the mycelial growth of all the strains tested **(Goudjil, 2016)**. Camphor has been shown to be characterized by antibacterial, antidysenteric and fungicidal activity **(Tantaoui-Elaraki *et al.*, 1993).** In addition, hydrocarbon monoterpenes and oxygenated monoterpenes of essential oils were capable of destroying cellular integrity, resulting in inhibition of respiration and impaired permeability **(Cox *et al.*, 2000).** Indeed, α-pinene, β-pinene and limonene inhibit respiratory activity in the yeast mitochondria **(Tepe *et al.*, 2005).**

5. Conclusion

Faced with the concern of patients who often suffer from superficial and especially recurrent candidiasis, our study was considered as a first promotion of extracts from plants in order to develop new antifungal substances unknown by our organism, due to the increase in phenomenon antibiotic resistance. The ointment was found powerful against *Candida albicans* strains. However, this preparation was qualified as non-irritating on dermal route.

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Declaration of interest

The authors report no conflicts of interest.

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