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PREPARATION, CHARACTERIZATION AND *IN VITRO* ANTIMICROBIAL ACTIVITY EVALUATION OF THYME ESSENTIAL OIL FORMULATION TO TREAT SKIN INFECTIONS

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ABSTRACT

The aim of the present study was to develop and evaluate a topical formulation containing *Thymus fontanesii* Boiss. & Reut essential oil that could serve as an antimicrobial agent for the treatment of various skin diseases caused by bacteria, yeast and dermatophytes. The selected formulation exhibited a significant antimicrobial activity against all tested strains, better consistency, good homogeneity, no skin irritation and good stability for six months at 25 ± 2°C. Our results demonstrate that the formulation could be used safely as topical preparation to treat skin diseases caused by the tested microorganisms into the future.

Keywords: Thyme; essential oil; antimicrobial activity; skin irritation; cutaneous infections.

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1. INTRODUCTION

Microbial skin infections especially bacterial, fungal and dermatophytes are very common and threatening in many cases[1]. Another significant concern is the development of resistance to the antibiotic in clinical use. For this reason, it is important to develop novel antimicrobial drugs, which can act against the microorganisms causing skin diseases[2].The search for new drugs of natural origin with antimicrobial action is an important focus of research worldwide. Plants from the Lamiaceae family have been widely used as spices or drugs, particularly due to their essential oils (EOs) used traditionally for many diseases concerning gastrointestinal, nervous, rheumatic disorders and skin diseases[3,4]. Within this family, the genus Thymus has received particular attention due to their aromatic composition and their biological properties[5]. In Algeria, there are nine (09) endemic Thymus species, most of them widely used as herbal remedies[6]. *T. fontanesii* Boiss.et Reut. is one of these endemic Thymus, known locally as “Zaitra” are perennial aromatic shrubs extensively used as folk medicine in the form of powders, infusions and decoctions for the treatment of many ailments including respiratory, digestive tube disorders[7,8]. Previous studies showed that the EO of *T.fontanesii* was active against the microorganisms causing various infectious diseases but these was any review study according the application of this EO or its formulation in the treatment of superficial mycosis by dermatophytes.

The objectives of the present study was to develop and evaluate an effective topical formulation containing *T.fontanesii* essential oil that could serve as an antimicrobial agent for the treatment of various skin diseases caused by bacteria, yeast, dermatophytes, assess its physicochemical properties and skin irritation. Evenly, the stability prepared formula was studied during six months.

2. RESULTS AND DISCUSSION

2.1 Yield and Chemical composition of the essential oil

The volatile oil extracted from *T.fontanesii* aerial parts (leaves and flowers) was pale yellow with pleasant perfumery odor.The extraction yield was about 3.5 ± 0.1% (v/w), which is higher than those found by Haddouchi et al.[9] and Mohammedi et al.[10] (2%, 3.09% respectively), for the same species of thyme harvested in Mostaganem and Tlemcen area

(Provinces located in the west of Algeria). Furthermore, it was higher than other species of thyme from Algeria, such as *Thymus lanceolatus*with 0.9% [11] and *Thymus dreatensis*with 2.1% [6].

A total of 17 compounds were identified by GC/MS comprised 99.58% of the essential oil. The identified components and their percentage (>0.01%) limits in each oil type are given in Table 1, where the components are listed in order of their elution from the HP-5MS column.

Table 1. Chemical composition of *Thymus fontanesii* essential oil

Compounds	**RI**[1]	**Percentage (%)**
Gamma-terpinene	1060	1,23
Thymol	**1290**	**44,31**
Carvacrol	**1298**	**34,98**
Copaene	1378	0,74
Alpha-gurjunene	1412	4,57
Beta-Caryophyllene	1416	2,63
Aromadendrene	1439	0,52
Alpha-humulene	1449	0,75
Allo-Aromadendrene	1462	0,88
Gamma-Muurolene	1469	0,89
Germacrene D	1485	0,63
Ledene	1490	1,02
Beta-Bisabolene	1509	2,54
Gamma-Cadinene	1513	0,56
Delta-Cadinene	1520	2,05
Alpha-Bisabolene	1532	0,59
Heptacosane	2700	0,61
Total identification%		**99,56**

Very high percentage of monoterpenes characterize the essential oil; this fraction represented

[1]RI: retention index, obtained with reference to an n-alkyne series C8H18–C20H42 using DB-5 column and the Van den Dool and Kratz equation.

more than 89% of total oil, especially the phenols ones (79.29%), thymol (44.31%) was found to be the major component of the essential oil, followed by carvacrol (34.98%), confirming that *Thymus fontanesii* is a thymol chemiotype, according to literature data for this species in Algeria from the province of Constantine, Djelfa and Setif area, dominated by thymol (68.2%, 29.3% and 67.8% respectively)[7,8,12]. This fraction followed by sesquiterpene fraction (19.05%). Only 1.23% of the volatiles constituents were monoterpene hydrocarbons.

Obviously, it is well known that the yield and the chemical composition of the EO can vary within the same species. Such variability depends on several factors including local climatic and environmental conditions, season, geographical location and geology [13,14].

2.2 Characterization of EO formulation and stability tests

The experiments showed that test 4 gives good results, that is to say 20 g of glycerol are sufficient to facilitate the spreading of the formulation on the skin and the relative presence of the large quantity of water is necessary for the dissolution of active ingredients, such as sodium tetra borate, $CuSO_4$ and salicylic acid (partially) and its exhibited better quality characteristics (better consistency).

The freshly selected formulation prepared (F4) was homogenous, greenish in color due to nature and appearance of bentonite. Their odor was characteristic related to thyme EO and showed good consistency (Thick enough without presence of lumps at spreading). These results are shown in Table 2.

Table 2. Stability studies of EO formulation

Parameters	**Months**			
	0	2	4	6
Color	No change in color			
Odor	No change in odor			
Consistency	Good			
Homogeneity	Homogenous			
Phase separation	No			
pH	5,6 ± 0,05	5,71± 0,15	5,87 ± 0,20	5,9 ± 0,12

No change in color, odor, homogeneity and consistency was observed for this formulation

after six (06) months of stability testing. These indicate that the ingredients and their amounts in formulation had been appropriately selected and the formulation had been sufficiently homogenized when prepared[15,16]. The pH values were found to be in the range of 5.6–5.9 from the time of preparation up to six months of the study period, which lies within the normal pH range of the skin[16]. Generally the pH formulation must be constant during storage time and its fluctuations can indicate complications such as microbial growth, ingredient incompatibilities or decomposition of some ingredients[17].

2.3 Skin-irritation test

Visual inspection assessed the results of skin irritation. No one animal indicates erythema or edema formation after 72 h of EO formulation application (Figure 1). The primary irritation index (PII) for the formula was found 0.29 which fell under the categories of no irritant indicates the safety of EO formulation[18].

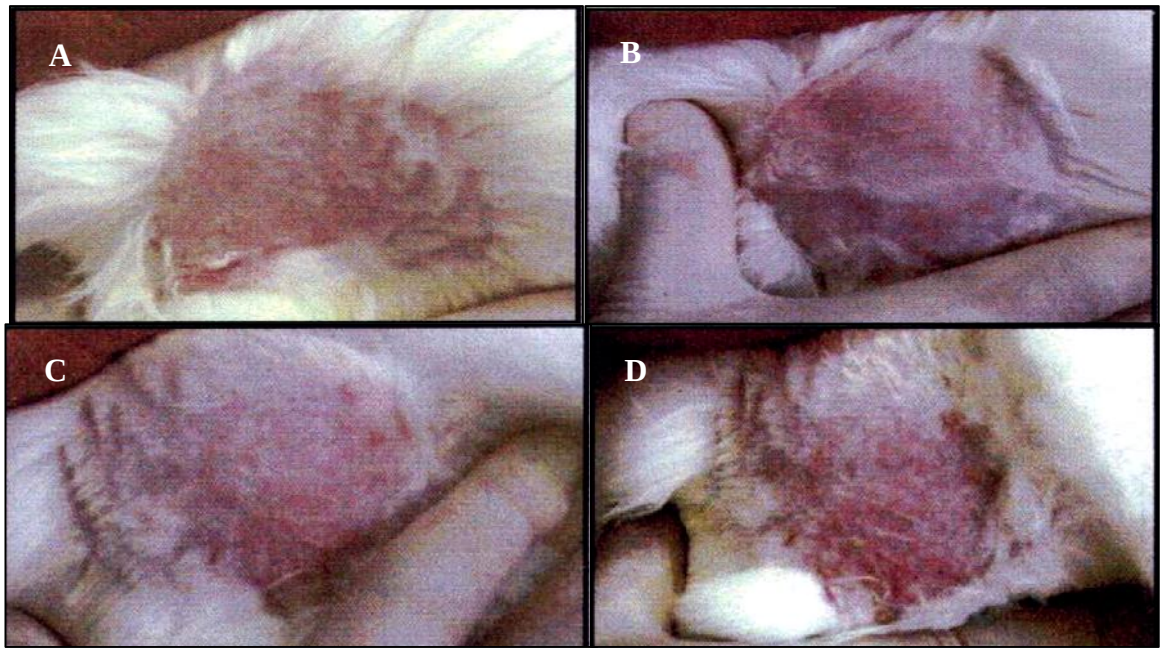

Fig.1. Photographs of rabbit skin treated with EO formulation: after 24 hours (A) intact and (B) abraded and after 72 hours (C) intact and (D) abraded

Results of the study clearly revealed that the selected formulation F4 is found to be stable after six months of the study with no skin irritation.

2.4 Antimicrobial activity

T. fontanesii essential oil and formulation prepared showed varying antimicrobial activity

degrees against all selected strains. They had noteworthy antimicrobial potential against all microorganisms tested, particularly against dermatophytes. The inhibition zones diameter (D) were in the range of 13.33–90 mm (Table 3).

The prepared formulation based on essential oil has a powerful inhibitory activity on all the bacterial strains tested whose mean values of the inhibition zones exceed 37 mm**.** It also has a very strong antifungal activity against all tested strains; it exhibited completely *T. mentagrophytes* and *M. canis*.There was significant difference between comparisons to marketed formulation (mecocide, mecotine, terbinafine) ($p < 0.001$) and control formulation without EO ($p < 0.01$) (Table 3, Figure 2 and Figure 3).

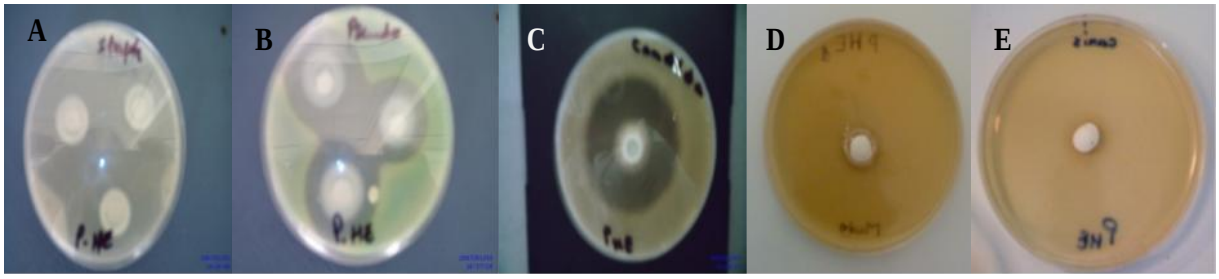

Fig.2. Antibacterial and antifungal activity of prepared formulation of *T.fontanesii* EO on: A. *S. aureus*, B. *Ps. aeruginosa*, C. *C. albicans*, D.*T.mentagrophyte, E. M.canis*

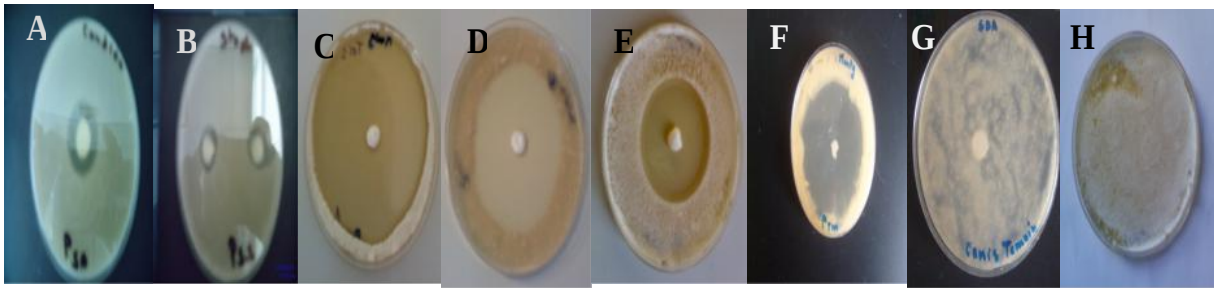

Fig.3. Antifungal and Antibacterial activity of mycotine on *C. albicans (A)*, mycocide on *S. aureus* (B), Terbinafine on: *T.mentagrophyte(C), M.canis (D),* formulation without EO on: *C. albicans (E), T.mentagrophyte (F),* witness : *M.canis (G), T.mentagrophyte (H).*

Table 3: Antimicrobial activity of *Thymus fontanesii* essential oil and EO formulation by the disc-diffusion method

Strains testded	Diameter (mean ± SD) of inhibition zone (mm)					
	EO[1]	**EO formulation**	**F. without EO**	**Mycocide**	**Mycotine**	**Terbinafine**
Bacterial strains						
Staphylococciesaureus (ATCC 6538)	$58,53^{***} \pm 0,83^{A}$	$59,50^{***} \pm 0,61^{A}$	$42,53 \pm 0,55^{B}$	$21,16 \pm 0,94^{C}$	$0,00^{D}$	-
Bacillus subtilis (ATCC 9372)	$43,36^{***} \pm 0,99^{B}$	$57,55^{***} \pm 0,64^{A}$	$40,53 \pm 0,65^{C}$	$24,28 \pm 1,04^{D}$	$0,00^{E}$	-
Enterococcus faecalis (ATCC	$33,19^{***} \pm 1,18^{C}$	$56,15^{***} \pm 0,28^{A}$	$39,70 \pm 0,49^{B}$	$12,41 \pm 0,72^{D}$	$0,00^{E}$	-
Escherichia coli (ATCC 4157)	$37,33^{***} \pm 0,59^{A}$	$39,66^{***} \pm 0,22^{A}$	$22,66 \pm 0,65^{B}$	$0,00 \pm 0,00^{C}$	$0,00^{C}$	-
Pseudomonas aeruginosa	$13,33^{**} \pm 0,80^{C}$	$34,04^{***} \pm 0,15^{A}$	$20,63 \pm 0,77^{B}$	$0,00 \pm 0,00^{D}$	$0,00^{D}$	-
Yeast						
***Candida albicans (ATCC* 24433)**	$47,06^{***} \pm 0,12^{B}$	$56,40^{***} \pm 0,60^{A}$	$41,11 \pm 1,88^{C}$	$12,20 \pm 1,23^{E}$	$21,50 \pm 0,81^{D}$	-
***Saccharomyces cereviseae (ATCC* 2601)**	$60,75^{***} \pm 0,65^{B}$	$69,05^{***} \pm 0,23^{A}$	$43,67 \pm 1,11^{C}$	$33,13 \pm 1,85^{D}$	$27,66 \pm 0,88^{E}$	-
Dermatophytes						
Microsporum canis	$90,00^{***} \pm 0,00^{A}$	$90,00^{***} \pm 0,00^{A}$	$66,47 \pm 0,71^{B}$	-	-	$56,01 \pm 1,04^{C}$
Trichophyton mentagrophytes	$90,00^{***} \pm 0,00^{A}$	$90,00^{***} \pm 0,00^{A}$	$77,36 \pm 1,13^{B}$	-	-	$74,67 \pm 1,23^{C}$

[1] SD: standard deviation, EO : T. fontanesii essential oil, F. without EO : formulation without EO.

Statistically significant differences compared to control are marked with different letters.

(*) at $p > 0.01$: not significant. (**) at $0.001 \leq p \leq 0.01$: highly significant. (***) at $p \leq 0.001$: extremely significant.

According to the values of the inhibition zones diameter (Table 3), and to the classification

established by Djabou*et et al.*[19], all the strains tested showed sensitivity to essential oil and formulation based on EO tested. Statistical analysis of products effect on bacteria allowed us to classify them according to their sensitivity: *S. aureus* > *B. subtilis* > *E. coli* > *E. faecalis* > *P. aeruginosa*. According to Ballester-Costa *et al.*[20], essential oil of *T.fontanesii* has a robust inhibitory activity against all pathogenic bacteria tested (D > 30mm) except on *P.aeruginosa* withmoderately inhibitory activity.(12 < D < 21mm).This bacterium has resistance to many essential oils and antibiotics drugs[11,21]. This could be explained by the structure of envelope cell [21]. It also has a strong antifungal activity against all strains tested; it exhibited completely *Trichophyton mentagrophytes* and *Microsporum canis* at 10 µL of the oil. Similar results were shown by Pinto*et al*.[22]who reported that the *Thymus pulegioides*oil was effective as an antifungal agent against dermatophytes, Aspergillus and Candida species. These results are following the earlier published data [5, 21].

Although the minimum inhibitory concentrations (MICs) and minimum bactericidal–fungicidal concentrations (MBCs–MFCs) values of the EO of *T.fontanesii* against the tested strains were in the range of (0.1–10 µl/ml) (Figure 4), in the most cases the MICs was equivalent to the MBCs - MFCs, indicating a bactericidal or fungicidal activity of this oil on all strains used in this study, according to Adrar *et al*.[23] and Hajlaoui *et al*. [24], the essential oil exercises a bactericidal effect when MBC/MIC ≤ 4.

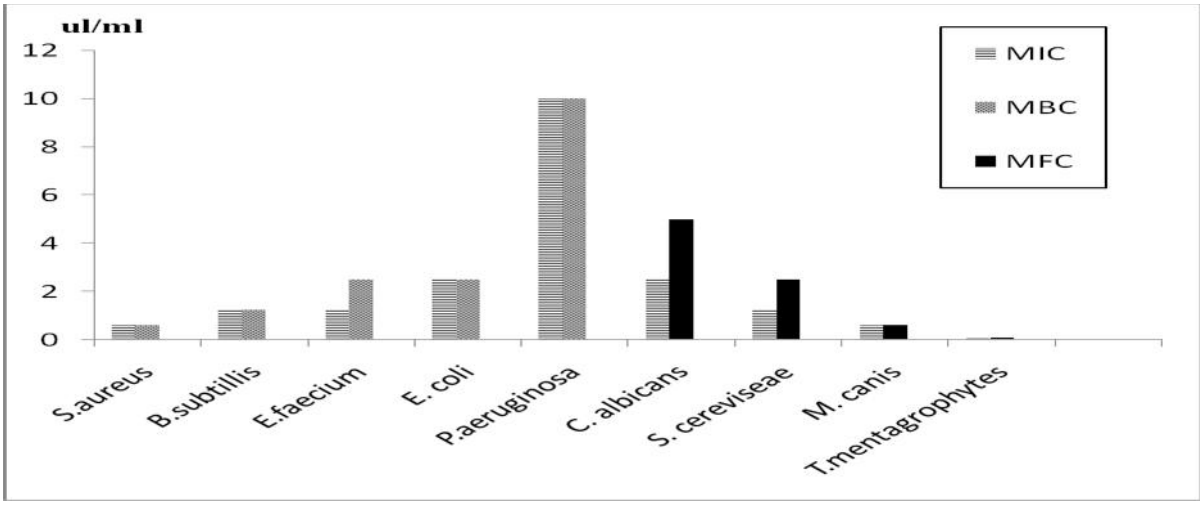

Fig.4. MIC, MBC and MFC of *T. fontanesii* essential oil

The high antimicrobial activity of *T.fontanesii* essential oil has been attributed to its phenolic

components such as thymol and carvacrol[21, 23]. Thymol and its isomer carvacrol, the major components of thyme EO, can alter the outer bacterial membrane[20,25].They combine with the charged groups of bacterial membranes, thereby increasing their permeability, and they lead to electrolyte and cellular ATP leakage[21, 25].

The higher antimicrobial activity of EO formulation was related to their chemical composition. Moreover, complex interactions between essential oil components and other chemicals utilized could be responsible for synergistic effects. These critical points are to be confirmed in further experiments.

3. EXPERIMENTAL

3.1 Materials

Glycerol, Zinc oxide, salicylic acid, sodium tetra borate, cupric sulfate ($CuSO_4$), formaldehyde and Tween 80 were obtained from Sigma-Aldrich (St. Louis, MO, USA), whereas bentonite was supplied from Bental (Algeria). All the chemicals utilized were of the best quality available in the laboratory. (The purity of the ingredients was 99.9%).

3.2 Experimental Animals

Six adult male white New Zealand rabbits weighing 2.5–3 kg were housed in standard environmental conditions.The experimental protocol was approved by the Animal Care and Use Research Committee of Research Center SAIDAL, Algeria.

3.3 Plant Material and Extraction of Essential Oil

The aerial parts (flowers and leaves) of *T. fontanesii* were collected during the flowering period from Lakhdaria mountain at 1040 m altitude (Bouira region), 74 km south east of Algiers. A voucher specimen was deposited in the herbarium of the Department of Botany, National Institute of Agronomy (INA), Algiers. The essential oil was obtained by the steam distillation for 3 using a Clevenger-type apparatus and stored in sealed glass vials at 4 °C in the dark until used.

3.4 Analysis of Essential Oil

Gas chromatography-mass spectroscopy (GC-MS) analysis of the oil was carried out on an Agilent HP-6890 gas chromatograph with a HP-5MS 5% phenylmethylsiloxane capillary column (30 m - 0.25 mm, 0.25 µm film thickness) coupled to a quadrupole mass spectrometer

(model Agilent HP 5973) network mass selective detector in the electron impact mode (Ionization energy: 70 eV). Analytical parameters were the following: the carrier gas was helium at a flow rate of 1 mL/min, the oven temperature was programmed from 60 to 280°C at 2 °C/min and held isothermal for 30 min. Injector and detector temperatures were set at 250 and 280 °C, respectively, and in the GC–MS analyses, temperatures of the ion source and transfer line were 170 and 280 °C, respectively. The Identification of the components was made by visual interpretation, comparing their retention indices and mass spectra with those stored in the mass spectrometry data base and with given in the literature by Adams terpene library[26-28].

3.5 Preparation of EO formulations

The essential oil formulation was prepared according to the reference document[29] with some modifications of the chemical composition. Briefly, substitute talc, plumb acetate, and menthol by bentonite, cupric sulfate **(**$CuSO_4$) and *T. fontanesii* EO respectively to minimize the side effects of these chemicals. The solid ingredients were mixed in a 250 ml flask. The glycerin was added gradually to the mixture before the addition of distillate water. To have a better formula consistency, it was taken different amounts of glycerol and water. Finally, the EO was added and stirred to produce homogeny formulation (Table 4). These prepared formulations were stored in glass vials sealed at room temperature in the dark until use.

Table 4. Composition of EO formulations

Ingredients	**F1 (%)**	**F2 (%)**	**F3 (%)**	**F4 (%)**
Glycerol	10	25	15	20
Distillate water	20	5	15	10
Bentonite	35	35	35	35
Zinc oxide	25	25	25	25
Salicylic acid	3	3	3	3
Sodium tetra borate	5	5	5	5
Formaldehyde	x	x	x	x
Cupric sulfate	0,5	0,5	0,5	0,5
***T. fontanesii* EO**	**x**	**x**	**x**	**x**

The selected formulation was subjected to stability study for six months at room temperature (25°C ± 2°C). The appearance, odor, color and phase separation of the formulations were visually evaluated, the formulation consistency was assessed by rubbing between the fore and first fingers. The pH of EO formulation was determined by using the pH meter and noted at various intervals for 6 month[30].

3.6 Antimicrobial activity

3.6.1 Microbial strains

The *in vitro* antibacterial activity of the essential oil from *T. fontanesii* and the selected formulation were tested against three Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 9372), *Enterococcus faecalis* (ATCC 6569),two Gram-negative bacteria: *Escherichia coli* (ATCC 4157), *Pseudomonas aeruginosa* (ATCC 9027), two yeast: *Candida albicans* (ATCC 24433), *Saccharomyces cereviseae* (ATCC 2601), obtained from the R.C.D (Research and Development Center, SAIDAL Algiers) and two dermatophytes strains obtained from Parasitology Laboratory, Centre Hospitalo-Universitaire Mustapha, Algiers, Algeria: *Microsporum canis* 4865,*Trichophyton mentagrophytes* 5221.

3.6.2 Antimicrobial screening of EO and EO formulation

The essential oil and formulation prepared were screened for antimicrobial activity using the disc diffusion method. Briefly; a suspension (0.2 mL of 10^6-10^8 CFU/mL) of each microorganismwas spread on Muller-Hinton agar (MHA) (bacteria), Sabouraud (yeast) and Sabouraud dextrose agar (SDA) (dermatophyte). Sterile filter paper discs (6 mm in diameter) were individually impregnated with 10 µL of essential oil and 1 mg/disc of formulation prepared, and then placed on the inoculated plates. The treated Petri dishes were incubated at 37°C for 24 h for bacteria, 25°C for 48 h for the yeast and at 27 °C during 7-14 days for the dermatothyte. Before incubation, all plates were stored in the dark at 4°C for 2h, to allow the diffusion of the oil from disc to medium without microbial growth. Also, Standard creams: mycotine, mycocide, terbinafine and formulation without EO (1 mg/disc) were used as positive and negative controls, respectively. The diameters of inhibition zones were measured in millimeters (including disc diameter of 6 mm). All the tests were performed in triplicate.

3.6.3 Determination of the minimum inhibitory concentration and minimum bactericidal–fungicidal concentration.

The broth macrodilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal–fungicidal concentration (MBC–MFC). The tests were performed in Mueller Hinton Broth (MHB) (bacteria), Sabouraud broth (yeast) and Sabouraud dextrose broth (SDB) (dermatophytes) supplemented with 5% Tween 80 at 2%. Tubes of MHB, SB and SDB containing various concentrations of oil were inoculated with 10µl bacterial or fungal inoculums adjusted to 10^6CFU/ml. The test tubes were incubated at 37°C for 24 h for bacteria, 25°C for 48 h for the yeast and at 27 °C during 7-14 days for the dermatothytes. The positive control was performed in the medium containing only the microorganism suspension. For determination of MBC or MFC, 0.1 ml of culture medium was a spirited from each micro broth assay tube showing no apparent growth and sub-cultured in fresh MHA (bacteria), Sabouraud (yeast) and SDA (dermatophytes). After incubation, the least concentration showing no visible growth on sub-culture was taken as the MBC-MFC. All tests were performed in triplicate.

3.7 Skin irritation test

The irritation effect of the selected EO formulation was evaluated by carrying out the Draize patch test on six rabbits[31]. These animals were kept in different cages and supplied with fresh food and water during the test period. The animal's backs were shaved 24 hours before the application of the formulation. The skin was observed for any visible changes such as erythema (redness) and edema (swelling) after 24 and 72 hours from the application of the formulation (0.5 g)for intact and abraded skin for all rabbits.[32]These reactions were evaluated according to the scoring system for skin reactions (ranging from 0 to 4)[18].

The primary Skin irritation index (PII) was calculated based on the sum of the scored reactions divided by 24 (2 scoring intervals multiplied by 2 test parameters multiplied by 6 rabbits).[32]The irritation degree was categorized as Non-irritant (0-0.4), or slight (0.5-1.9), moderate (2- 4.9) or severe irritation (5-8) based on the PII[18].

3.8 Statistical analysis

All experiments were performed in triplicate, and the data were expressed as mean ± standard

deviation (SD). The student's *t*-test and One way analysis of variance (ANOVA) followed by Post hoc Tukey test was applied to compare means and to analyze significance of the differences between antimicrobial activity. All analyses were performed using XLStat software (XLStat, Paris, France). p values<.05 were reported as significant.

4. CONCLUSION

Our results showed that the prepared formulation containing the essential oil of *T.fontanesii* possess strong antimicrobial activity against the selected microorganisms better than the marketed formulations. Furthermore, it had acceptable physical properties, excellent stability over six month of storage and no skin irritation. We believe that the formulation containing *T.fontanesii* EO may have a potential for further application as safe antimicrobial topical preparation to treat skin infections caused by the tested microorganism particularly dermatophytosis and mucocutaneous candidiasis.

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