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Study on antimicrobial activity of the essential oil of *Achillea maritima* L collected from Oued Righa beach, the North East of Algeria

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ABSTRACT

In this work we tested the antibacterial and the anti yeast activity of the essential oil extracted from the plant Achillea maritima L., harvested from Oued Righa, Filfla, the state of Skikda, Algeria, towards seven bacterial strains of Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and a yeast strain of Candida albicans. The content of total polyphenols in the plant methanolic exctract is 27.75 mg EGA / g methanolic extract whereas the rate of total flavonoids is 11.33 mg EQ/g methanolic extract. The essential oil exerted a weak antibacterial activity on Staphylococcus aureus showing an inhibition diameter zone of (9±0.27 mm), the rest of microbial strains was totally resistant to the tested essential oil. The minimal inhibitory concentration showed that the tested microbial strains could grow on all the dilutions. Our Achillea maritima L. essential oil has a weak antimicrobial activity, thereby it is recommended to do the extraction of essential oil using methods other than the hydrodistillation.

***Keywords*:** *Achillea maritima* L.*;* Antibacterial activity; Aromatogram; Essential oil; Minimal inhibitory concentration.

INTRODUCTION

Many bacterial strains have developed mechanisms of antibio-resistance. Therefore it is very necessary to search new antimicrobial agents from different sources (Chebaibi *et al*., 2016). Plants are a potential source of antimicrobials in many parts of the world (Alviano *et al*., 2009). Medicinal plants have been recognized for their use in traditional medicine practices since prehistoric times (Mirihagalla and Fernando, 2021). At the end of the XIXe century and the beginning of the XXe century many scientific research works have been related with the action of antiseptic actions of many essential oils (Chebaibi et *al*., 2016; Karaalp *et al*., 2009). People around the world, wildly use the medicinal plant *Achillea* L. in the treatment of various diseases (Yener *et al*., 2020). *Achillea maritima* L. is a species that lives in the maritime sands of the North of Algeria. It is an aromatic perennial herb, densely white lanate, with stems up to 50 cm, ascending, stout and woody. Leaves are 5–17 mm, oblong to oblong- lanceolate, entire or crenulate, fleshy and sessile. Inflorescence corymbose, with several medium capitula. It was reported to exhibit many biological ethnopharmacological activities such as the treatment of wound healing, bladder inflammation, wound healing, dysentery and digestive disorders like diarrhea and gastric gas (Lee *et al*., 2019; Muselli *et al*., 2007). It is also used as a decorating plant which has an effect against flying insects (Christodoulopoulou *et al*., 2005)

Our research aims to test the possibility of using the essential oil extracted from the plant *Achillea maritima* L. collected from Oued Righa beach, the state of Skikda, the North East of Algeria, in the treatment of some microbial infections caused by multiresistant bacteria and yeasts. It is the first time that the antimicrobial activity of the plant variant growing in Algeria was revealed.

MATERIALS AND METHODS

Vegetal material

The aerial parts of the plant (Fig.1) were collected in January 2021 from Oued Righa beach,

the town of Skikda, the North East of Algeria whose geographical coordinates are 36.9025, 6.8779. The plant was identified by Dr. Sakhraoui N., a botanist in the university of August 20th, 1955-Skikda. The collected parts were then dried and stocked in a dry and dark place

Extraction of essential oil

It was done by the hydrodistillation method, using a clvenger apparatus. 20 grams of the plant were cut into small pieces and placed in a ball of 500 ml impregnated with distilled water. The whole was boiled during 2 or 3 hours. Vapors charged with essential oil, traversing a refrigerant column, condenses in a clean beaker. The distillate was then decanted to separate the organic phase charged with essential oil from the aqueous phase. Water particles in the extracted essential oil were eliminated by adding magnesium sulfate. Pure essential oil was then preserved at 4°C, away from light, in small dark glass bottles (Makhloufi *et al*., 2012). The extraction yield was calculated using the following formula of Harbone (1998):

Extraction of oil yield= weight of the extract/ weight of the vegetal material) x 100.

Preparation of the methanolic extract

The aerial parts of the plant washed and dried at room temperature were grinded. Ten grams of the powder were macerated in methanol 70% and continuously stirred for 24 h. The obtained solution was applied onto a filter paper and poured into Petri dishes to be dried at 30°C for 48h. The obtained methanolic extract was used for the determination of total polyphenols and total flavonoids.

Dosage of total polyphenols

The concentration of polyphenolic content was determined using the method of Folin-Ciocalteu (Singleton, 1999). 1ml of Folin-Ciocalteu diluted 10 times, was added to 0.2 ml of the methanolic extract solution previously diluted in dimethyl sulfoxide (DMSO) at a concentration of 1mg/ml. 0.8 ml of Na_2Co_3 (7.5%) were then added to the mixture. The whole was incubated at room temperature during 2 hours. The absorbance of the mixture was measured using a spectrophotometer at 765nm, against a blanc without methanolic extract. The concentration of polyphenols was calculated from a linear calibration curve (y = ax + b) previously created with different concentrations of Gallic acid (0-1000mg/ml). The concentration of polyphenols was expressed in milligram of equivalent Gallic acid per gram of methanolic extract (mg EGA/g methanolic extract) (Boizot and Charpentier, 2006).

Dosage of total flavonoids

The concentration of total flavonoids was measured using the colometric method of aluminum chloride with some modifications (Brighente *et al*., 2007). 1 ml of the methanolic extract solution diluted in DMSO at a concentration of 1mg/ml as well as the dilutions of quercetin standard solution (20, 40, 60, 80 and 100 ug/ml) were added to 1 ml of $AlCl_3$ (2 % w/v). After an incubation period of 40 minutes at room temperature, the absorbance was measured at 430 nm against a blanc without methanolic extract. The concentration of total flavonoids was determined from a linear calibration curve of quercetin (y = ax + b). It was expressed in milligramg equivalent quercetin per gram of methanolic extract (mg EQ/g methanolic extract) (Bahorun *et al*., 1996).

Bacterial strains

Antimicrobial activity of *Achillea maritima* L. essential oil was tested against seven bacterial strains and a yeast strain. The pathogenic strains (02 strains of *Escherichia coli*, 01 strain of *Klebsiella pneumoniae*, 01 strain of *Pseudomonas aeruginosa* and 01 strain of *Staphylococcus aureus*) were provided by the laboratory of RENAD clinic, Skikda and the microbiological laboratory of Abderrazak Bouhara hospital, Skikda. The type strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were obtained from the hospitalian center of Louis Giorgi, Orange-France. *Candida albicans* ATCC 10231 was provided by the microbiological laboratory of the faculty of sciences, Skikda University.

Re-isolation of the pathogenic bacterial strains

To confirm the purity of the microbial strains, they were re-cultured in Hektoen medium for *Escherichia coli* strains and *Klebsiella pneumoniae*, nutrient agar for *Pseudomonas aeruginosa*, Chapman medium for *Staphylococcus aureus* strains and Sabouaud agar for *Candida albicans* ATCC 10231.

Evaluation of essential oil antimicrobial activity (Aromatogram)

The sensitivity of the bacterial strains towards *Achilllea maritima* L. essential oil was tested using the agar medium direct contact technique (Aromatogram) according to the following steps.

Preparation of mother solution

Essential oil was added to distilled water to obtain a mother solution concentration of 2560 ug/ml (Khadri , 2009). Tween 80 (0.05%) and ethylic alcohol 95% (2% v/v) were added to the mother solution to get a homogenate solution.

Preparation of standard inoculums

Re-isolated bacterial strains were firstly transplanted and incubated at 37° C for 18-24h. The yeast strain was incubated at 28 °C for 18-24h. The bacterial inoculums was prepared by diluting 2 or 3 separated colonies in physiological water to get a suspension of 0.08-0.1 absorbance read at 625 nm (0.5Mc Farland) (CA-SFM, 2012). The yeast suspension was standardized at an absorbance of 0.08-0.1 read at 449 nm.

Seeding

Muller Hinton medium was firstly poured in sterile Petri dishes. After solidification, the medium was seeded with the bacterial inoculums using a sterile swab. For the yeast strain Muller Hinton medium was replaced by Sabouraud agar. 6 mm sterile Wattman filter disks loaded with 10 ul of the essential oil mother solution were deposited aseptically. A control disc loaded of distilled water, tween 80 and ethylic alcohol was added as control disc. The plates were then incubated at 37° for 24h. The trial was performed in triplicates and the antimicrobial activity of the essential oil was evaluated after calculating the mean of the inhibition zones diameters as:

not sensitive (≤ 8 mm), sensitive (9-14 mm), very sensitive (15-19 mm) and extremely sensitive (≥ 20mm) (Moreira *et al*., 2005).

Preparation of mother solution and determination of minimal inhibitory concentration (MIC)

It was determined by the agar dilution method. Serial dilutions of the microial mother solution from 2560-10 ul/ml were prepared according to the method of dilution in geometric progression at a rate of 2 (1/2-1/256) (Khadri, 2009). In Petri dishes, 1 ml of each dilution was mixed with 9 ml of Muller Hinton or Sabouraud melted agar. After homogenizing the mixture, Petri dishes were inoculated with the same suspension prepared for the Aromatogram. The bacterial plates were incubated at 37°C for 24-48 h and the yeast plate at 28°C for 18-24h. The MIC was determined by the first concentration that inhibits the microbial growth.

Antibiogram of the tested strains

The sensitivity of the tested bacterial strains to *Achillea maritima* L. essential oil was compared with their sensitivity to the used antibiotics. Antibacterial susceptibility (Antibiorgam) of the bacterial strains to the tested antibiotics was determined according to the Comity of the Antibiogram-French Society CA-SFM (2010). Bacterial inoculums of 0.5 Mac Farland, an equivalent of 10^6 cells/ml, was prepared. Muller-Hinton medium was then seeded by the bacterial inoculums using the swabbing technique to get tight streaks. The antibiotic disks were deposited o the surface of the medium and the plates were incubated at 37 °C for 24 h. The bacterial strains were classified as resistant or sensitive depending on the inhibition zones obtained around the antibiotics disks (Comity of the Antibiogram-French Society CA-SFM, 2012).

Statistical analysis

The diameter of the inhibition zones was calculated in triplicates. The obtained values were presented as mean± Standard Deviation (SD) (Steel *et al*., 1997).

RESULTS AND DISCUSSION

Yield of essential oil extraction

The obtained essential oil was characterized by a blue color and a characteristic smell. The yield of extraction was 0.08%. It was superior than that of *Achillea maritima* L. collected from Corsica (0.02-0.06%) (Muselli *et al*., 2007). It was however weaker than the yield obtained from the essential oil of the same species collected from Portugal (0.3–0.4%) (Cabral *et al*., 2013). Comparing our results with those of El-Kalamouni *et al*. (2017) who worked on *Achillea millefolium*, we found that our

yield is very close to theirs (0.07%). Our results are inferiors than those of Lamamra (2018) who reported that the essential oil extracted from *Achillea santolinoides* harvested from Djelfa, Algeria was 2.19%. Hatem and his collaborators (2018) showed that the essential oil of *Achillea fragrantissima* gathered from the North of Bekaa-Lebanon, had a yield of 1.25%. The yields of essential oils were different despite the use of the same technique of extraction (Hydrodistillation). This is explained by the geographic origin of the plant, the environmental factors of the plant chemotype (Bruneton, 1993). The temperature, the rate of humidity, the soil composition and the sunshine duration are the main factors that affect the chemical composition of essential oils. Fadil *et al*. (2015) proved the effect of time of hydrodistillation and the drying duration on the maximization of essential oil yield.

Rate of total polyphenols

The polyphenols content was calculated from the linear calibration curve of Gallic acid whose equation is $y = 0.004x + 0.024$ with $R^2 = 0.996$ (Table1). The obtained results indicate that the rate of polyphenols in *Achillea maritima* L. was 27.75 mg EGA/g methanolic extract. This rate is inferior than that recorded by Haliloglu and his collaborators (2017), who reported that the methanolic extracts of flowers and leaves of *Achilleae sivasica* are characterized by polyphenolic rates of 54.7 and 51.1 mg EGA/g extract respectively. They are however superiors of the polyphenolic contents of flowers and leaves aqueous extracts of the same plant (22.7 and 17.9 mg EGA/g extract respectively) (Haliloglu *et al*., 2017).

Rate of total flavonoids

The total flavonoids content was calculated from the linear calibration curve whose the equation is $y = 0.009x + 0.048$ with $R^2 = 0.998$ (Table 1). We obtained a rate of 11.33 mg EQ/g methanolic extract. The recorded rate is less than the rate recorded by Haliloglu and his collaborators (2017) in the methanolic extract of leaves and flowers of *Achilleae sivasica* (31.4 and 27.1 mg EQ/g extract respectively). Our result was however superior than the total flavonoids concentrations found in the aqueous extract of leaves and flowers of *Achilleae sivasica* (4.4 and 3.7 mg EQ/ g extract respectively) (Haliloglu *et al*., 2017). It was noted that the method of extraction and of quantification may influence the rate of total polyphenols (Lee *et al*., 2003) as well as the genotypic and geographic differences (Khadri, 2019). The temperature applied during the hydrodistillation has a negative effect on the rate of polyphenols. The alcoolic extract however increases the extraction of polyphenols by increasing permeability of the cell wall and facilitating the extraction of polyphenols (Seidel, 2006). The plant physiological state during the harvest also affects the rate of polyphenols (Khadri, 2019).

The sensitivity of bacterial strains to the tested antibiotics

The obtained results (Table 2A & Table 2B) reveal that the strain of *Staphylococcus aureus* was resistant to 50% of antibiotics. *Escherichia coli* isolated from urine was resistant to 30% of antibiotics. The strain of *Escherichia coli* isolated from diabetic foot was however resistant to 56% of the tested antibiotics. *Pseudomonas aeruginosa* was characterized by a high rate of resistance (67%). The type strains were sensitive to all the applied antibiotics (100%). Most of the clinical bacterial strains are resistant to the applied antibiotics (Silver, 2011). Chromosomal mutations, transmission of resistance genes by conjugative plasmids, phages or transposons are the main causes of the bacterial antibio-resistance. This resistance leads to the research of new antimicrobial substances more efficient than the synthetic drugs and more tolerated by the human organism (Garcia-Ruiz *et al*., 2008; Kempf and Zeitouni, 2009).

Evaluation of antimicrobial activity of essential oil of *Achillea maritima* L.

The obtained results (Table 3 & Fig. 2) indicate that the extracted essential oil was active only towards *Staphylococcus aureus* which showed an inhibition zone of (9±0.27 mm) (Moreira *et al*., 2005). On the other hand, the clinical strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and the type strains of *Escherichia coli ATCC* 25922, *Staphylococcus aureus* ATCC 29213 and *Candida albicans* ATCC

Table 1: Rate of total polyphenols and total flavonoids in the methanolic extract of *Achillea maritima* L.

	Concentration of total polyphenols (mg EGA/g methanolic extract)	Equation of the curve	R^2
Methanolic extract	27.75	y = 0.004x + 0.024	0.996
	Concentration of total flavonoids (mg EQ/g methanolic extract)	**Equation of the curve**	**R²**
	11.33	y = 0.009x + 0.048	0.998

Table 2A: Sensitivity of the tested Enterobacterial strains and *Pseudomonas aeruginosa* to the tested antibiotics

Bacteria **Antibiotics**	*Eshcerichia coli* isolated from urine	*Escherichia coli* isolated from diabetic foot (pus)	*Escherichia coli* ATCC 25922	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
COT	R	R	S	R	/
AMX	R	R	S	R	R
CZ	S	R	S	R	S
FO	S	S	S	S	R
CIP	S	R	S	S	S
AMC	S	R	S	R	R
NIT	S	S	S	S	S
FOX	/	/	/	/	R
P	/	/	/	/	R
TE	/	S	S	/	/
AMP	/	S	S	/	R

Table 2B: Sensitivity of the tested staphylococci strains to the tested antibiotics

Bacteria **Antibiotics**	***Staphylococcus aureus***	***Staphylococcus aureus*** **ATCC 29213**
COT	/	/
AMX	/	/
CZ	/	/
FO	/	/
CIP	/	/
AMC	/	/
NIT	/	/
FOX	R	S
P	S	S
TE	S	S
AMP	R	S

S:sensitive, R: resistant, / not tested

COT: Cotrimoxazole, AMX: Amoxicilline, CZ: Cefazolin, FO: Fosfomycine, CIP: Ciprofloxacine, AMC: Amoxicilline + acide clavulanique, NIT: Nitrofurantoin, FOX: Cefoxitine, p: Pénicilline, TE: Tetracycline, AMP: Ampicilline

Table 3: Sensitivity of the microbial strains to the essential oil of *Achillea maritima* L. (Aromatogram)

	Aromatogram	
Microbial strain	**Inhibition zone (mm)**	**Sensitivity**
***E. coli* (pus)**	6 ± 0.43	-
***E. coli* (urine)**	6 ±0.5	-
***E. coli ATCC* 25922**	6 ±0.3	-
S. aureus	**9 ±0.27**	+
***S. aureus* ATCC 29213**	6 ±0.1	-
K. pneumonia	7 ±0.5	-
P. aeruginosa	6 ±0.35	-
***C. albicans* ATCC 10231**	6 ±0.35	-
Control disc	**6 ±0.5**	-

- not sensitive (≤ 8mm), + sensitive (9-14 mm)

10231 grew without any inhibition power around the essential oil discs. The obtained inhibition zones were 6± 0.1-7±0.5mm. Cabral and his collaborators (2013) in turn showed weak antimicrobial activity of *Achillea maritima* L. against the same tested species of *Candida* (*C. albicans* ATCC 10231). The results of El-kalamouni *et al*. (2017) on *Achillea millefolium* collected from France are also in agreement with ours. They recorded an inhibion zone of 9.1 mm against *Staphylococcus aureus* and no antimicrobial activity was found against *Escherichia coli.* Daniel *et al*. (2020) also reported a weak antimicrobial activity of *Achillea millefolium* L. essential oil against *Escherichia coli, Staphylococcus epidermidis Candida. albicans* and *Klebsiella pneumoniae. Achillea fragrantissima* was however characterized by a considerable antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter faecalis*, *Salmonella enteritidis, Pseudomonas aeruginosa* and *Candida albicans* with inhibition zones of 45 mm, 40mm, 38mm, 31mm, 30mm and 15mm respectively (Hatem *et al*., 2018). Previous studies confirmed that essential oils have pronounced antimicrobial activities against Gram positive bacteria in comparison with Gram negative bacteria. This is awarded to the external hydrophilic membrane of Gram negative bacteria which prevents the penetration of hydrophobic compounds to the targeted cell (Wan, 1998).

Minimal inhibitory concentration of essential oil of *Achillea maritima* L.

The obtained results were confirmed quantitatively by the Minimal Inhibitory Concentration (MIC) in solid medium (Fig. 3 & Fig. 4). The tested strains grew at all the concentrations (1/2-1/256). Our results are in contradiction with those of El-Kalamouni et *al.* (2017) which revealed that essential oil of *Achillea millefolium* L. was characterized by a minimal inhibitory concentration of 120ug/ml against *Staphylococcus aureus*. Yener *et al*. (2020) confirmed that *Achillea vermicularis* of Turkey exerts a strong antimicrobial activity against *Escherichia coli* with a minimal inhibitory concentration of 30ug/ml. Essential oil of *Achillea millefolium* collected from Bresil showed a minimal inhibitory concentration of 30 mg/ml against *Escherichia coli, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Candida albicans* (Daniel *et al*., 2020).

The essential oil of *Achillea maritima* L. collected from Oued Righa the state of Skikda, Algeria did not show a significant antimicrobial activity. This is due to many factors among which we cite the low concentration of polyphenols known for their antimicrobial effects like sabinene, 1,8-cineole and the camphor (Pattnaik, 1995), the loss of essential oil volatile compounds during the storage and the extraction (Chemloul, 2014), the low diffusion of the extracted essential oil into the agar and the period of collection.

Fig.1:Aerial parts of *Achillea maritima* L. collected from Oued Righa, Skikda

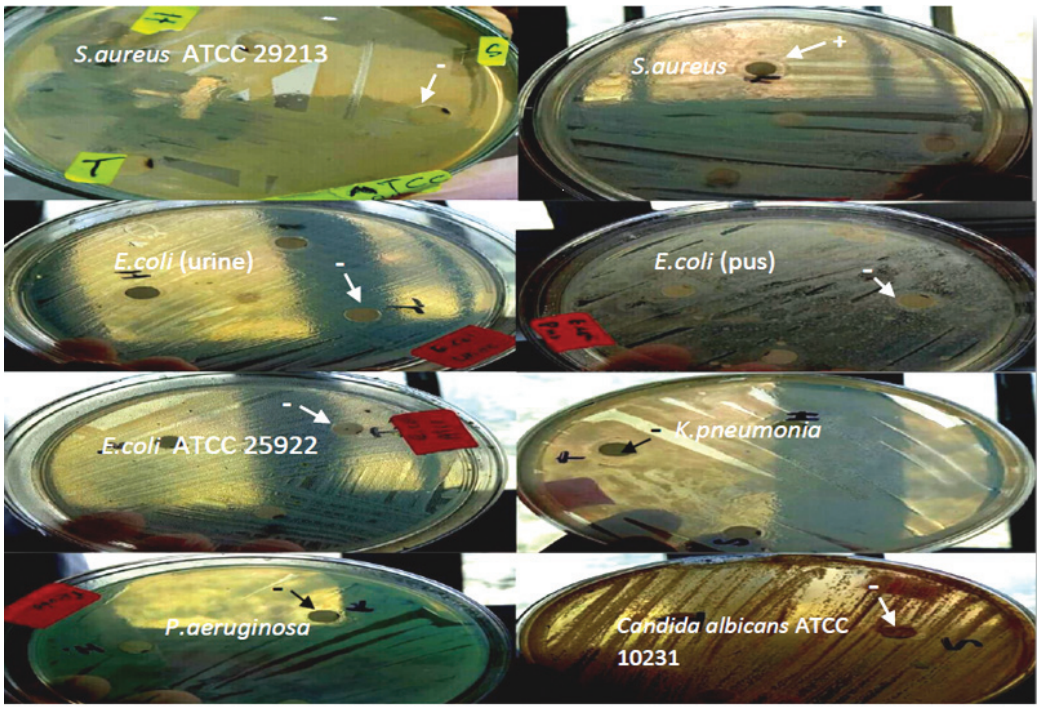

Fig. 2: Antimicrobial activity of *Achillea maritima* L. essential oil using the method of Aromatogram
- not sensitive (inhibition zone ≤ 8mm), + sensitive (inhibition zone 9-14 mm)

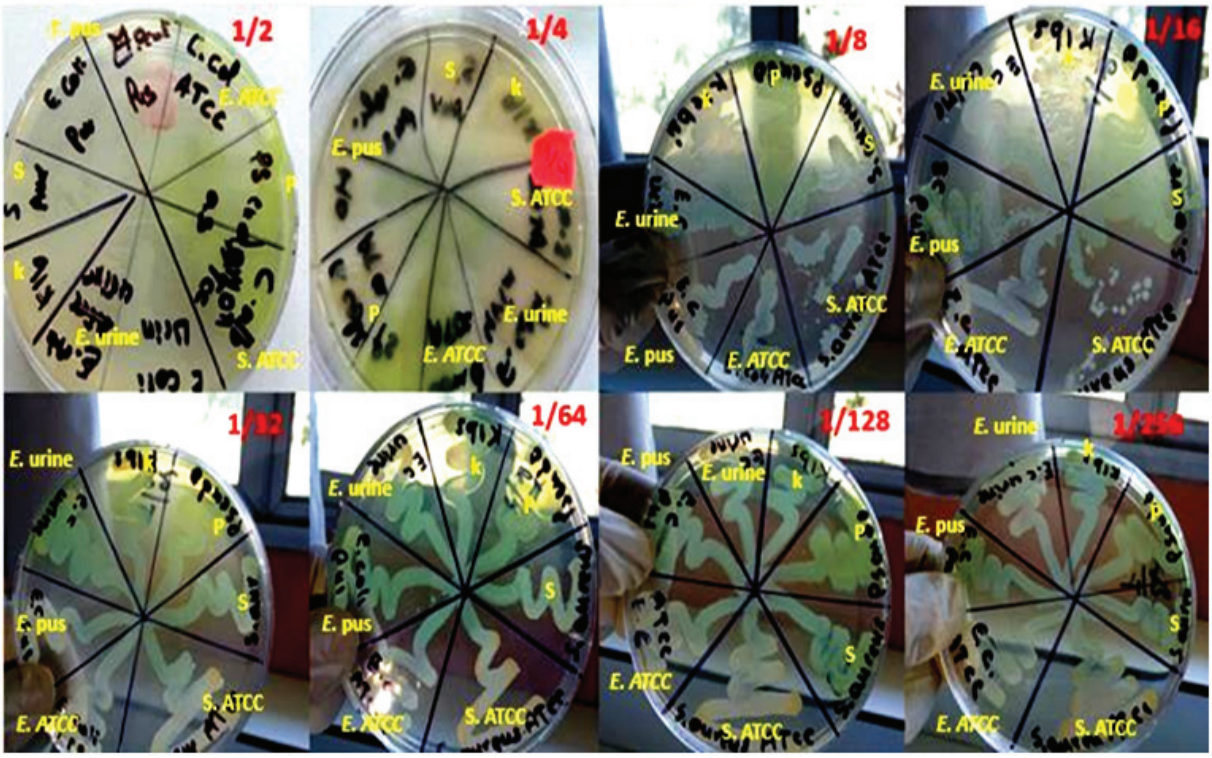

Fig. 3: Minimal inhibitory concentration of *Achillea maritima* L essential oil towards the tested bacterial strains.
E.pus: *Escherichia coli* isolated from pus, E.urine: *Escherichia coli* isolated from urine, E. ATCC: *Escherichia coli* ATCC 25922, K: *Klebsiella pneumonia*, P: *Pseudomonas aeruginosa*, S: *Staphylococcus aureus*, S. ATCC: *Staphylococcus aureus* ATCC 29213

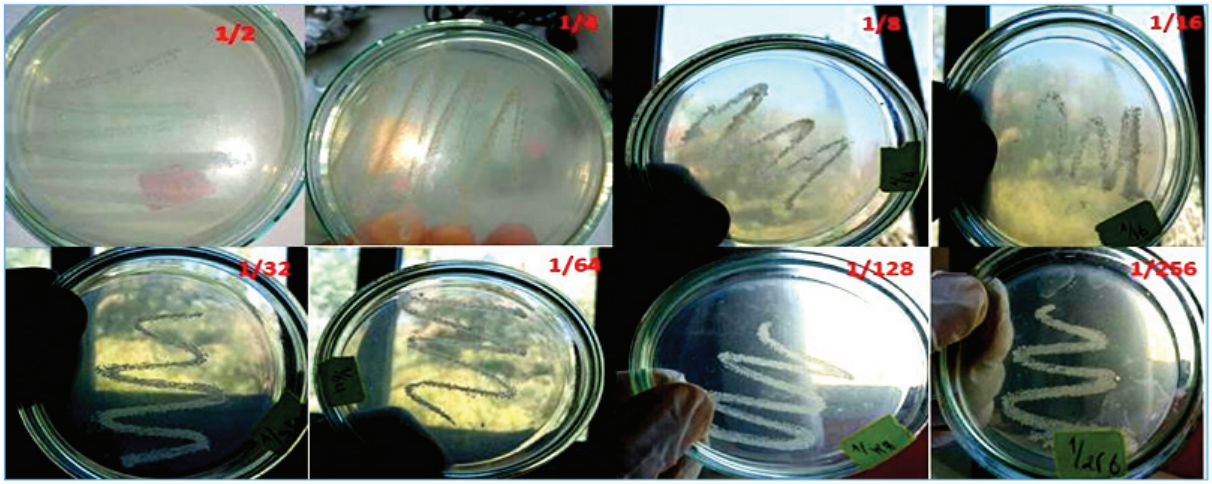

Fig. 4: Minimal inhibitory concentration (MIC) of *Achillea maritima* L. essential oil towards the tested *Candida albicans* ATCC 10231

Gas chromatography analysis showed that the essential oils of *Achillea maritima* L. aerial parts collected from different beaches in Portugal are mainly composed of chrysanthenone (40.4–57.2%), filifolone (12.2–15.5%), cischrysantenyl acetate (10.1–12.2%) and alpha-pinene (6.7–7.2%) (Cabral *et al*., 2013). Romeo *et al*. (2007) revealed that the major volatile components extracted from *Achillea maritima* L. aerial headspace, collected from Messina (Italy) and analyzed by gas chromatography-Mass spectrometry, are santolina triene (24.35%), camphor (17.91%) and artemisyl acetate (13.19%). Gas chromatography and gaz chromatography-mass spectrometry analysis of the oils obtained by hydrodistillation of the headspace parts of *Achillea maritima* L. growing in Greece, showed that the major components of the oils are chrysanthenyl acetate (30.4%); camphor (12.9%) and artemisia alcohol (12.6%) (Tsoukatou *et al*., 2000). In fact, an essential oil chemical composition varies according to the plant vegetative cycle (Khadri, 2019) in addition to the plant chemotype.

CONCLUSION

The sensitivity of some microbial strains towards the essential oil extracted from *Achillea maritima* L. collected from Oued Righa the state of Skikda Algeria was tested for the first time. The studied essential oil exerts a weak antimicrobial activity. *Staphylococcus aureus* is the only sensitive bacterial strain to the action of *Achillea maritima* L. essential oil. The obtained results of the aromatogram were confirmed by the minimal inhibitory concentration where the strains could grow at all the concentrations (1/2-256). Thereby it could be interesting to take in consideration other aspects among which:

- The extraction of essential oil using methods other than the hydrodistillation
- The extraction, the purification, the identification and the dosage of active components
- Testing the antimicrobial activity towards other pathogenic microbial strains.

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