Polyphenols, Flavonoids content and Antibacterial, Acetylcholinesterase Inhibitory Activities of *Ammodaucus leucotrichus* Coss. & Dur. Fruit from Algerian Sahara

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ABSTRACT: This study describes the quantity of the polyphenols, the flavonoids, and the antibacterial activity of different fruit extracts from *Ammodaucus leucotrichus* Coss. & Dur. subsp *leucotrichus* which is an Algerian endemic species. The quantitative estimation of total phenols and flavonoids was performed using the colorimetric method (dosage with Folin Ciocalteu's and aluminum trichloride reagent) and showed that the methanol extract is rich in these compounds. The antibacterial activity and the minimal inhibitory concentration (MIC) of the plant extracts against eight bacterial strains tested (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumonia* ATCC 13883, *S. typhimurium* ATCC 13311, *P. vulgaris* ATCC 13315, *B. cereus* ATCC 14579, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29912) were assessed using the disk diffusion method. The antibacterial activity results showed that the methanolic extract (EM1) is higher than the antibacterial potential of the reference antibiotic (Céfalexine) against *E. faecalis*. The Inhibitory Minimal Concentration (MIC) recorded, varies from 1/4 mg/ml to 1/16 mg/ml for all the investigated bacterial strains. Therefore, the extracts and the fractions of *A. leucotricnus* fruits were found to be active with a bacteriostatic effect for the different bacterial strains. The methanol extract from *A. leucotrichus* fruit assessed an acetylcholinesterase average inhibiting activity.

KEYWORDS: *Ammodauchus leucotrichus*, fruit, polyphenols, flavonoids, Antibacterial, MIC and bacteriostatic effect.

I. INTRODUCTION

Several searchers all over the world show that plant medicinal have always occupied an important place in medicine. These natural substances from plants have multiple interests. They are exploited in the biotechnology industries, in food, cosmetics and pharmaceutical. These compounds include a large proportion of secondary metabolites which are illustrated in many areas and even in therapy such as the compounds polyphenolic, steroids, alkaloids and terpenes [1]. Many works showed that the secondary metabolites have an important potential as antibacterial, antifungal, antioxidant, and ant diabetic agents, etc. If the discovery and the use of antibiotics were at the origin of the greatest successes of medicine, today there is enough scientific evidence on the existence of increased resistance of bacteria to antibiotics. Therefore, the search for new natural agents with less danger on health and overcoming the antibiotics side effects has become a necessity. As part of the search for natural antibacterial substances, we have tested several bacterial strains of *A. leucotrichus;* a plant employed in traditional therapy in Africa. *Ammodaucus leucotrichus* Cosson & Durieu is from *Leucotrichus* subsp. and the Apiaceae family. It is a small glabrous annual plant locally named "Nessoufa" and known as Cumin chevelu in French. The plant is characterized by streaked stems and small shaped branches, fleshy leaves finely divided forming flat narrow ridges, umbels with 2 to 4 rays, and involucres having very divided bracts, the white flowers are positioned according to a composed umbel. The mericarps are 6-9 x 4-5mm long with secondary ribs covered with 8 to 10 mm long silky hair which is very dense and fuzzy with a yellow-reddish base and a white end [2].

A. leucotrichus is an aromatic plant endemic of North Africa (Algeria, Morocco, Tunisia, Libya, Egypt, Mauritania, and upper Niger valley). Its best implantation is in desert regions, often down a hill or a dune [3], [4]. The plant is used in traditional medicine and the fruits are prepared through decoction to heal diabetes, cold, fever, and stomachache particularly with children [5]. In Morocco, flowers are used to heal heart disease [6] .Several researchers have reported some pharmacological properties of this plant. In studies carried out by Abu Zarga *et al* and Gherraf *et al* [7], [8], essential oils of *A. leucotrichus* fruits show antimicrobial activity against microorganisms including antibiotic-resistant bacteria and fungi. Another study revealed the aqueous extract protective effect of *A. leucotrichus* fruits against the urinary lithiasis tested *in vitro* [9]. The main objective of

this work is to evaluate the antibacterial activity (bactericidal and/or bacteriostatic action) of the various extracts and fractions from *A. leucotrichus* fruits on growth in vitro of eight bacterial stocks.

II. MATERIALS AND METHODS

Chemicals and Reagents : Acetylthiocholinesterase (AChE), Bovine serum albumin,1-naphthyl-acetate, Fast blue B and the TLC plates Silicagel 60 F_{254} were purchased from Merck (Germany), Gallic acid and catechin, Folin-Ciocalteu reagent, $AlCl_3$ (aluminum trichloride), Dimethyl ether, $CHCl_3$, CH_2Cl_2, Ethanol, methanol, n-butanol, (Na_2CO_3) sodium carbonate, ($NaNO_2$) Sodium nitrite, NaOH, ethyl acetate, (DMSO) dimethyl sulfoxide, Distilled water, lead acetate, Muller Hinton medium (MHI), and Sulfate anhydrous sodium were obtained from Sigma-Aldrich Chemicals. Other used chemicals and reagents were of analytical grade.

Collection of Plant Samples : The plant material consists of *A. leucotrichus* Cosson & Durieu fruits harvested in the region of Bechar (Southern Algeria). Our plant was authenticated by Professor A. Marouf (Institute of Science and Technology, Department of Natural Sciences and Life, University of Naama, Algeria). The dried fruits were pulverized through a crusher to get a fine powder. The Powder was used to prepare different extracts.

Preparation of extracts

- **Extracts (EM1, EM2, EE 70%, EED and EA):** These extracts were obtained using the method described by Haddouchi *et al* [10]. Five milligrams of vegetable powder from fruits were extracted with 50 ml each of methanol 80%, ethanol 70%, dimethyl ether and aqueous individually at ambient temperature with agitation for 24–48 hours. The five extracts obtained (EM1, EM2, EE70%, EED and EA) were concentrated under reduced pressure and the residue containing water was dried by lyophilization and stored at 4 °C in the dark.
- **Extracts (DCM, AcOET and BOH):** Twenty-five grams of the ethanolic extract 70% were included in 200 ml of distilled water-added with lead acetate [$(CH3COO)_4Pb$] to remove chlorophyll and other low molecular weight compounds by precipitation. After filtration, the solution became red-brown. The filtrate was subjected to sequential extraction with dichloromethane, ethyl acetate and n-butanol. Thus, three obtained organics phases (DCM, AcOEt and BOH) were dried by sulfate anhydrous sodium, then filtered, concentrated, and dried under reduced pressure.
- **Essential oil:** The essential oil was obtained through 8 hours of hydrodistillation. Distilled plant material consists of *A. leucotrichus* dried fruits. Oil was dehydrated with anhydrous sodium sulfate and stored at 4 °C in the dark.

Determination of total phenolic : The methods for the quantification of total polyphenol content were based on colorimetric measurements. Some tests were relatively specific to polyphenols (for instance the Porter's assay). The total polyphenols rate in methanol extract of *A. leucotrichus* fruits was measured using the Folin-Ciocalteu reagent method [11]. Briefly, in test tubes a volume of 100 µl of methanol extract or standard solution of Gallic acid (0.01, 0.02, 0.03, 0.04, and 0.05 g/l) was added to 2 ml of Na_2CO_3 solution (2% dissolved in water). After 2 minutes, 100 µl of reagent Folin-Ciocalteu (50% dissolved in water) was added to the mixture. The tubes were agitated and preserved at room temperature for 30 min. The absorptance was read to 750 nm. Total phenolics content was expressed in milligram equivalent of Gallic acid per gram of extract (mg GAE/g).

Determination of flavonoids : The Flavonoid rate in methanol extract of *A. leucotrichus* fruits was determined using the method described by Kim *et al* [12]. Five hundred microliters of methanol extract were mixed with 1500 µl of distilled water followed by the addition of 150 µl of $NaNO_2$ (5% dissolved in water). The mixture allowed a reaction for 5 min. Following this, 150 µl of $AlCl_3$ (10% dissolved in water) was added and the mixture stood for a further 6 min. Finally, the reaction mixture was treated with 500 µl of 1M NaOH. The absorbance was measured at 510 nm and was obtained against blank prepared similarly by replacing the extract with methanol using a spectrophotometer. The concentration of the flavonoïds was deduced starting from a calibration curve established with catechin (10-50 µg/ml) and expressed in the equivalent micrograms of catechin per milligram of extract (mg EQ/g of extract).

Acetyl cholinesterase (AChE) inhibiting activity through bio autography : The inhibition of the acetyl cholinesterase by the extracts and the pure products was tested by bioautography on a thin layer according to the method developed by Marston *et al* [13]. The samples for the TLC analysis were prepared by dissolution of the extracts in methanol (10 µl). The samples were deposited on the TLC plates and eluted by a solvent system $CHCl_3$: MeOH: H_2O (65: 35:5). After being thoroughly dried, the plates were sprayed with acetylcholinesterase solution in 0.05 M Tris-HCl buffer at pH 7.8 containing bovine serum albumin to stabilize the enzyme (1000 U / 150 ml = 6.67 U / ml). After 20 min of incubation at 37 ° C in a humid atmosphere, the plates were sprayed with

a mixture (1: 4) of an ethanolic solution of 1-naphthyl-acetate (2.5 mg / ml) and a solution of Aqueous Fast Blue B salt (2.5 mg / ml). The active compounds were indicated by light spots on a purple background.

Antibacterial activity evaluation : The eight bacterial cultures used in this study are: three bacteria gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29912 and *Bacillus cereus* ATCC 14579), and five bacteria gram-negative (*Escherichia coli* ATCC 25922, *pseudomonas aeruginosa* ATCC 27853, *Klebsilla pneumonia* ATCC 13883, *Salmonella typhimurium* ATCC 13311 and *Proteus vulgaris* ATCC 13315). These bacterial cultures are of clinical origin (isolated and identified by the Bacteriology Service, Hospital EHU of Oran, Algeria).The anti-bacterial activity test carried out on the various fractions and the extracts of the fruits of *A. leucotrichus* Coss. & Dur was determined using the disk diffusion method [14], [15]. The bacteria species were cultivated for 24 h in Muller Hinton medium (MHI) at 37°C. The suspension of the tested microorganisms (approximately 10^6 CFU/ml) was spread on the solid media plates (20 µl). A sterile 6-mm-diameter Filter disk (Wattman paper N° 3) was impregnated with 10 µL of serial dilutions in dimethyl sulfoxide (DMSO) of the various fractions and the extracts (EM1, EM2, EE70%, EED, DMC, and BOH), and was placed onto the solid media plates. The inhibition diameter was measured after 24 h of incubation at 37 °C. The standard antibiotic CN (Céfalexine 10 µg) was used in order to control the sensitivity of the tested bacteria. The antibacterial activity was assessed by measuring the inhibition growth zone surrounding the disks.

Determination of Minimum Inhibitory Concentration (MIC) : MIC is the concentration or the amount of the antimicrobial agent inhibits visible growth of a microorganism. This parameter does not quantify the number of the bacterial population that was eradicated. The extracts and fractions having shown a positive antimicrobial activity were selected to determine the minimum inhibitory concentration (MIC). The modified method used by Usman and Osuji [16] was employed to determine the minimum inhibitory concentration. The different dilutions of *A. leucotrichus* fruit extracts and the fractions were prepared in dimethyl sulfoxide (DMSO) to yield 0,625-5 mg/ml. The suspension of the tested microorganisms (approximately 10^6 CFU/ml) was spread on the solid media plates (20 µl). The sterile 6-mm-diameter Filter disks (Wattman paper N° 3) was impregnated with 10 µL of the various fractions and the extracts (EM1, EM2, EE70%, EED, DMC, and BOH), and incubated at 37°C for 24 hours. The MIC value was determined at the same time as the lowest concentration of the extract in the broth medium that inhibited the visible growth of the test microorganism [17].

Determination of the effect bacteriostatic or bactericidal : The distinction between bacteriostatic and bactericidal agents was developed primarily from in vitro studies. The bacteriostatic effect refers to the agents that inhibit the bacteria growth but do not eradicate the bacteria, and bactericidal means that the agent kills the bacteria. In fact, the classification for an agent could be specific for particular laboratory conditions and bacterial strains. The determination of the bacteriostatic or bactericidal effect of our fractions and extracts was carried out by subculturing the MICs inhibition zones formed and which do not show any bacterial growth visible to the naked eye on a medium culture (HD). The cultures were then incubated at 37°C for 24 h.

III. RESULT AND DISCUSSION

Total polyphenols and flavonoids contents : Methanol extract quantitative study was processed through spectrophotometry. The purpose was to assess the total polyphenols and flavonoids contents. Gallic acid and catechin calibration curves of polyphenols and flavonoids respective dosing are recorded in Table 1.The quantity of polyphenols and flavonoids compounds of some species in Apiaceae family present the significant levels of these compounds. Our results are in agreement with the report of Rat *et al.*, [18] who showed the important levels of phenolics (from 74 to 120 mg TAE/g), and flavonoids (from 7.63 to 14.52mg RE/g) of three Apiaceae species: *Heracleum lasiopetalum Boiss, Kelussia odoratissima Mozaff* and *Echinophora platyloba DC.* We also noted that the results obtained by 25 who showed that the estimated phenols and flavonoids of *A. leucotrichus* fruits sample taken from Béchar region reported the important levels of phenolics (from 118.69 to 160.61 mg GAE/g DW), and flavonoids (from 87.76 to 97.38 mg QE/g DW).

Table1: The content of polyphenols, flavonoids and AChE inhibition in different extracts from *A. leucotrichus* fruits.

Samples	Total polyphenols			Flavonoids			AChE
	Equation of the trend	**coefficient R^2**	**[ ] (mg GAE/g)**	**Equation of the trend**	**coefficient R^2**	**[ ] (mg CE/g)**	
EM	**Y=0,059X**	**0,965**	**482,28±5,62**	**Y=0,108X**	**0,970**	**61,57±2,05**	+
EO							-
EA							-

EM: methanol extract, **EO:** Essential oil, **EA**: aqueous extract, **AChE:** acetyl cholinesterase inhibitory.

Acetyl cholinesterase (AChE) inhibiting activity : We also found that the methanolic extract of *A. leucotrichus* fruits exhibited a moderate acetyl cholinesterase inhibitory activity (Table1) compared to that found by [19] which is a negative result of the aerial part aqueous extract of this same plant.

Antibacterial activity : The results of the *in vitro* antibacterial susceptibility test of the various fractions and extracts of the fruits from *A. leucotricus* on the growth of eight bacterial strains are presented in Table 2 and Fig. 1. The quantitative comparison of the results from the compounds tested and the antibiotic is difficult because the nature of the activity and the chemical composition of the molecules are not completely comparables, one can, all the same, make a total comparison of the activity of antibiotics and compounds by the expression of the report of inhibition.

Table 2: The relative inhibition of the extracts tested compared to the positive witness (Céfalexine).

	Bacterial strain	**Samples [a]**						
		EM1	**EM2**	**EED**	**EE$_{70\%}$**	**BOH**	**DCM**	**CN**
Gram +	***S. aureus***	18.75	18.75	18.75	18.75	**53.125**	**56.25**	**100**
	E. faecalis	**>100**	-	**100**	-	**100**	**100**	**100**
	B. cereus	18.75	18.75	18.75	18.75	**53.125**	**56.25**	**100**
Gram -	***P. aeroginosa***	37.50	**56.25**	37.50	43.75	**56.25**	**68.75**	**100**
	E. coli	-	33.33	33.33	33.33	33.33	33.33	**100**
	K. planticola	19.44	25.00	22.22	44.44	38.89	44.44	**100**
	S. typhimurium	**50.00**	37.50	37.50	37.50	**56.25**	**50.00**	**100**
	P. vulgaris	28.57	25.00	28.57	25.00	25.00	25.00	**100**

EM1: Methanolic extract (24h); **EM2**: Methanolic extract (48h); **EED**: Dimethyl ether extract; **EE$_{70\%}$**: Ethanolic extract 70%; **DMC**: Dichloromethanic fraction; **BOH**: Butanic Fraction; **CN:** reference antibiotic (Céfalexine). **a**: relative inhibition %.

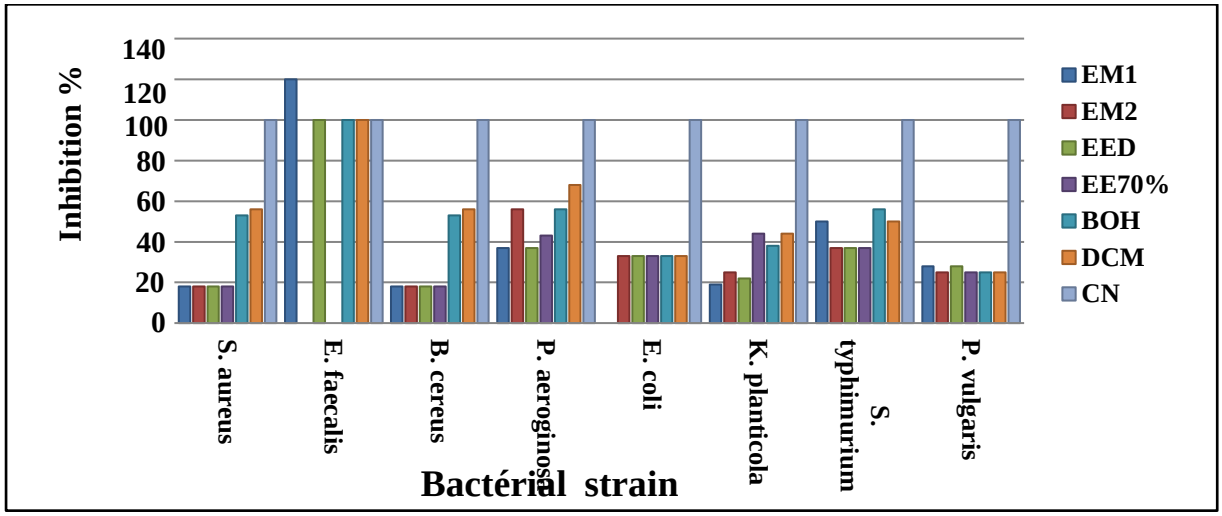

Fig. 1: The antibacterial activity of the extracts tested compared to the positive witness (Céfalexine).

The results of the tested extracts and fractions represent a relative inhibition which equalizes at 100% for EED and the two fractions (BOH, DCM) on the stock of *E. faecalis*. However, the methanol extract (EM1) has higher antibacterial potential than that of the reference antibiotic (Céfalexine) on *E. faecalis*. By comparison with Céfalexine, the two fractions (BOH, DCM) and two extracts (EM1, EM2) showed inhibitions higher than 50%

on the bacteria to gram-positive (*S. aureus*, *B. cereus*) and bacteria than Gram-negative (*P. aeroginosa*, *S. Typhimurium*). These two fractions showed a significant antibacterial activity against Gram-negative and Gram-positive bacteria. This activity could be explained by the presence of various components, in particular, the flavonoïdes and the total polyphenols which exist in the different extracts of the *A. leucotrichus* fruit.Their chemical structures can have an important role in the antibacterial activity by disturbing the cell structures making them more permeable, leading to cell death [20], [21], [22].Compared to other studies on the medicinal plant *Ammodaucus leucotrichus* Coss.& Dur tested on different extracts and essential oils it showed a good action on different bacterial strains. However, the work carried out on the essential oil of the *A. Leucotrichus* fruits *in vitro* by Gherraf *et al* and El Haci *et al* [7], [23] showed a significant antibacterial activity against the microorganisms examined (*Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*).

Determination of Minimum Inhibitory Concentration (MIC) : The results of the minimum inhibitory concentration are shown in Table 3 and Fig. 2. These results indicate that the MIC of the different extracts and fractions of *A. leucotrichus* fruits forms zones of inhibition on the medium evaluated in millimeters. The MIC of extract EE 70% and the two fraction ((BOH, DCM) against the bacteria tested (*S. aureus*, *B. cereus and K. planticola*) were 0.625 mg/ml, while it was 1.25-2.5 mg/ml for all other stocks bacterial. However, all extracts did not show any minimum inhibitory concentration activity against bacterial strain *E.colis*. The EM1 extract did not inhibit the growth of all the tested bacteria with the exception of *E. faecalis* with the MIC of 1.25 mg/ml.

Table 3: Minimum Inhibitory Concentration of different fractions and extracts of *A. leucotrichus* fruits.

Microbial strain	**Samples**																							
	EM1				**EM2**				**EED**				**EE 70%**				**BOH**				**DCM**			
	a	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**
S. aureus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**15**	**14**	**10**	**7**	**16**	**13**	**10**	**7**
E. faecalis	**8**	**7**	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. cereus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**16**	**12**	**9**	**7**	**16**	**14**	**10**	**7**
P. aeroginosa	0	0	0	0	**7**	0	0	0	0	0	0	0	0	0	0	0	**8**	**7**	0	0	**9**	**8**	0	0
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. planticola	0	0	0	0	**8**	**7**	0	0	**7**	0	0	0	**15**	**12**	**12**	**10**	**11**	**9**	**8**	**8**	**15**	**11**	**9**	**8**
S. typhimurium	**7**	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**8**	**7**	**7**	0	0	0	0	0
P. vulgaris	**8**	**7**	**7**	0	0	0	0	0	**7**	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

a=5mg/ml, **b**=2.5 mg/ml, **c** = 1.25mg/ml, **d** = 0.625mg/ml.

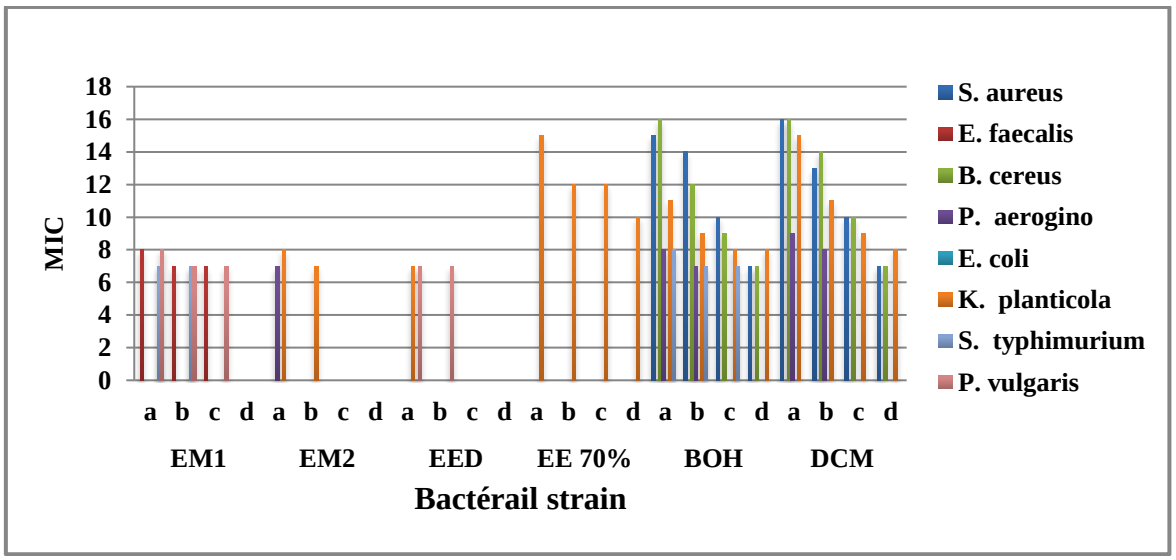

Fig. 2: Minimum Inhibitory Concentration of different fractions and extracts of *A. leucotrichus* fruits.

Opposite the bacteria with gram-positive, we note the CMI of extract EM1 and two fractions (BOH, DCM) about 1/4 mg/ml and 1/16 mg/ml representing a low diameter of inhibition of 7 mm against the bacteria *E.*

Faecalis, B. cereus and S. aureus, respectively. However, the bacteria with gram-negative present the CMI of 1/4 mg/ml and 1/8 mg/ml with diameters of inhibition of 7, 8 and 9 mm for the two fractions (BOH, DCM) and the three extracts (EM1, EM2, EED) against the bacteria *K. planticola, S. Typhimurium, P. aeroginosa and P. Vulgaris.*

Determination of the bacteriostatic or bactericidal effect : To confirm the bactericidal or bacteriostatic effect of the various fractions and the extracts of the *A. leucotrichus* fruits, the transplantation of the strains from the inhibition zones on nutrient agar was preceded against the bacteria *E. Faecalis, B. cereus, K. planticola, P. aeroginosa and P. Vulgaris* and *S. aureus* with different concentrations (5¸ 2.5¸ 1.25 and 0.625mg/ml). From the results of Fig. 3, we notice that all the tested extracts and fractions of *A. leucotrichus* fruits have a bacteriostatic effect opposite to all the studied bacterial strains.

Fig. 3: Test of activity bacteriostatic and bactericidal of the various fractions and extracts of *A. leucotricus* fruits on various studied bacterial strains.

1 -EM1: Methanolic extract (24h); **2 -EM2**: Methanolic extract (48h); **3 - EED**: Dimethyl ether extract; **4 - EE$_{70\%}$**: Ethanolic extract 70%; **5 - DMC**: Dichloromethanic fraction; **6- BOH:** Butanic Fraction; **a**=5mg/ml¸ **b**=2.5 mg/ml¸ **c** = 1.25mg/ml¸ **d** = 0.625mg/ml.

IV. CONCLUSION

Natural substances have shown great potential in the treatment of human diseases such as infectious diseases. In this context, We tested the antibacterial activity (*in vitro*) of the various extracts and fractions from *A. leucotrichus* fruits against different stocks bacterial (*E. Faecalis, B. cereus, K. planticola, P. aeroginosa, P. Vulgaris* and *S. aureus*). Our results provide basic information about the quantitative estimation of total phenols and flavonoids and antibacterial activity (bacteriostatic effect) of *A. leucotrichus* fruit that might be helpful in planning future pre-clinical experiments on this potent plant. Other additional research is necessary to characterize and identify the bioactive molecules present in all the extracts and fractions of the *Ammodaucus leucotrichus* Coss. & Dur. fruits; a plant used in the world and Algeria, in the south of the Sahara in particular as traditional remedies for some infections.

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